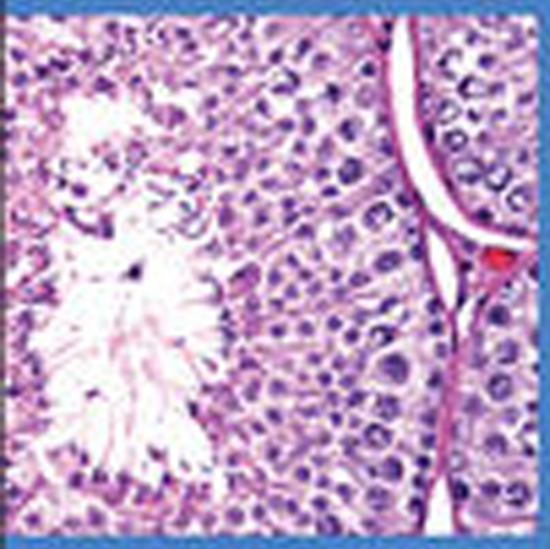
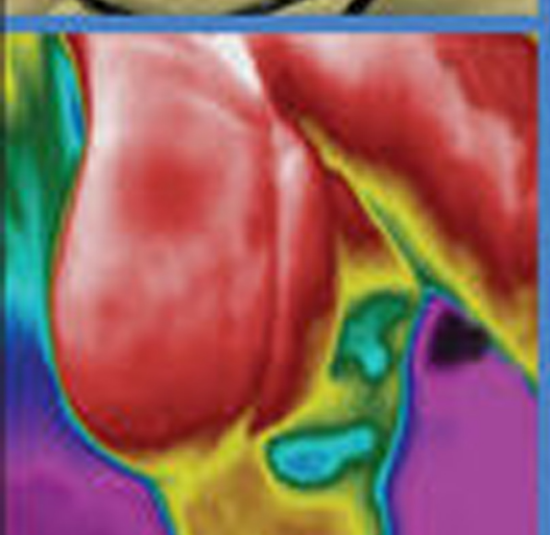
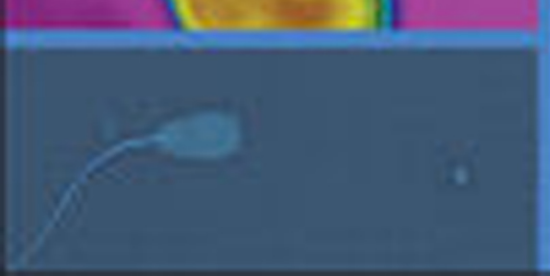






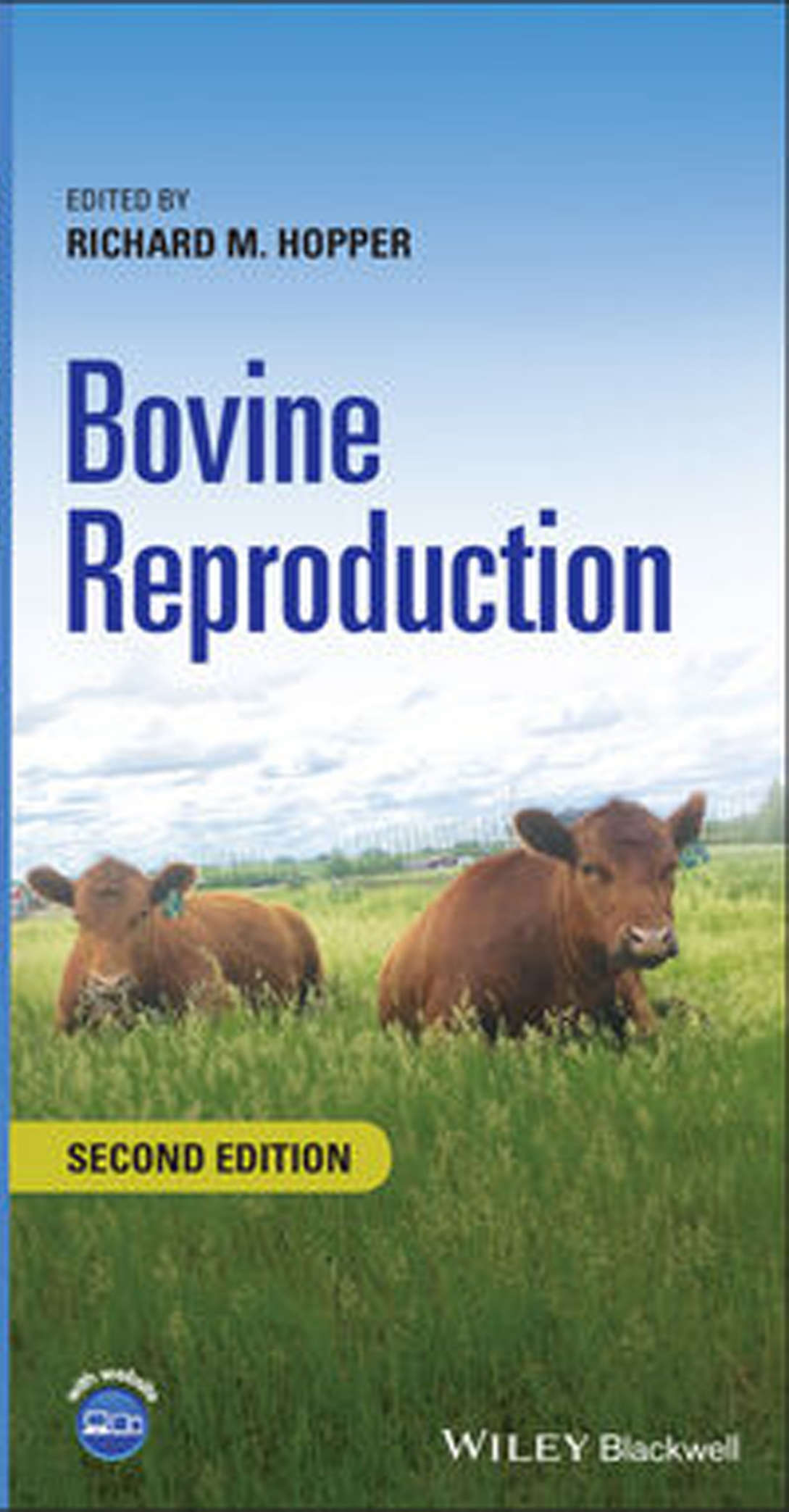




EDITED BY

RICHARD M. HOPPER

Bovine Reproduction



SECOND EDITION

WILEY Blackwell

Bovine Reproduction

"Let our cattle bear, without mishap and without loss"
Psalms 144:14, *New American Standard Bible*

Bovine Reproduction

Second Edition

Edited by

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WILEY Blackwell

This edition first published 2021
© 2021 John Wiley & Sons, Inc.

Edition History
© 2014 John Wiley & Sons, Inc.

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Registered Office
John Wiley and Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA

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111 River Street, Hoboken, NJ 07030, USA

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Library of Congress Cataloging-in-Publication Data

Names: Hopper, Richard M., 1954– editor.
Title: Bovine reproduction / [edited by] Richard M. Hopper.
Description: 2nd edition. | Hoboken, NJ : Wiley-Blackwell, 2021. | Includes bibliographical references and index.
Identifiers: LCCN 2020054589 (print) | LCCN 2020054590 (ebook) | ISBN 9781119602361 (cloth) | ISBN 9781119602453 (adobe pdf) | ISBN 9781119602460 (epub)
Subjects: MESH: Cattle–physiology | Reproduction | Reproductive Medicine–methods | Reproductive Techniques–veterinary | Pregnancy Complications–veterinary
Classification: LCC SF768.2.C3 (print) | LCC SF768.2.C3 (ebook) | NLM SF 768.2.C3 | DDC 636.2/1–dc23
LC record available at https://lccn.loc.gov/2020054589
LC ebook record available at https://lccn.loc.gov/2020054590

Cover Design: Wiley
Cover Image: © Richard M. Hopper; © Colin Palmer

Set in 9.5/12.5pt STIXTwoText by SPi Global, Pondicherry, India

10 9 8 7 6 5 4 3 2 1

To Donna *– a wonderful and patient wife who, while not completely sharing my love for cows, nevertheless became skilled at watching expectant heifers from the kitchen window with binoculars and herding escaped yearling bulls from the front yard.*

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Preface and Acknowledgments to the Second Edition

As I near completion of a task that I promised myself I would never repeat, I have to say that the singular aspect of this project that stands out to me is the incredible work of the contributors. Although a handful of chapters remained virtually unchanged, most received serious updates. Pregnancy wastage rightfully includes neonatal and calf at side loss, but a new section specifically focusing on the neonate was added. The importance of lameness and its impact on fertility necessitated its inclusion. Chapters that represent recent technological advances have been added as well. Due to the broad acceptance of the first edition, an intentional effort was made to recruit more authors from outside North America and I believe this provides a more international perspective for the manner in which we all approach reproductive management of the bovine. Much like the first edition, I again utilized a broad array of both veterinary specialists and animal scientists. I also believe this book benefits from the contributions of practicing veterinarians, a group often underrepresented in veterinary texts.

Thus, I must begin my thanks by acknowledging the tremendous contributions of the chapter authors and acknowledge the guilt I feel when someone refers to this as "my book." I also owe a profound debt of gratitude to the incredible staff at Wiley. Their competence and publishing standards are the reasons that we have such a good reference text. Specifically, Erica Judisch who first displayed the faith in me to shepherd this task and then again thought we should update it. Susan Engelken, though not as directly involved as with the first edition, was again a helpful asset on "the other end" of several e-mails. A special thanks to Merryl Le Roux, who came on midway through to serve as project editor and to see this to completion; and to both Julie Musk and Sandeep Kumar. Julie was the copyeditor who found the many errors that I had missed and also did an incredible job with the numerous suggestions that have made the text so much more readable; and Sandeep handled the final content refinement. I also appreciate my colleagues at both Mississippi State and Auburn for their support.

On a personal note, Joshua Lewis, Elizabeth Marian, and Lucas Michael have joined granddaughter Abigail Betty since the first edition came out and to quote my friend John Myers, "my children are only above average, but my grandchildren are exceptional." I also need to thank my wife Donna for her now 38 years of patience and support, but will refer you to the dedication for that.

A significant portion of my career (15 years) in practice was followed by 26 years at Mississippi State and finally the opportunity to return to Auburn, my alma mater. I feel that I have indeed led a charmed life and being afforded the opportunity to contribute to the profession and specifically the area of bovine theriogenology through this text has been a great blessing.

Auburn, Alabama *Richard M. Hopper*

Preface and Acknowledgments to the First Edition

There is an old fable in which three penniless and hungry travelers come to a small town. Unsuccessful in finding work or even a handout, one concocts a novel plan. He goes to the middle of the village carrying three fist-sized rocks and announces with great aplomb that he is planning to make his famous "stone soup." The skeptical but curious villagers gather. Well of course he needed a kettle and some water. The inquisitive villagers wondered if that was all. "Yes," he replied, "but it is better with a little garnish to improve the flavor." One villager thought that he could spare some carrots, another some potatoes, and a third some meat. This continued with virtually everyone in the village contributing. The result of course was a wonderful soup and everyone enjoyed a fine meal, while experiencing an object lesson in cooperation.

The story bears an ironic resemblance to the development of this text. The editor, like the plucky traveler, personally short on ability and resources but acutely aware of a need, enlisted the assistance of those who possessed both. Excellent reference texts were available on equine and small animal theriogenology, but a current bovine text was much needed. The goal was to produce a text that would service the needs of the veterinary student and bovine practitioner, as well as the graduate student and resident.

While I would readily admit that this text could be improved with respect to the choices made vis-à-vis the organization of the book or the order of some chapters, I honestly do not believe that I could have done any better than the contributors selected. The authors of this text represent a wide array of specialties and educational and experiential backgrounds. I will forever be grateful for their assistance and immensely proud of their individual contributions. I would also like to acknowledge the efforts of my graduate assistant, Amanda Cain, who in addition to contributing a chapter, prepared the glossary of terms and index. Likewise, I would like to thank everyone at Wiley for their help. Erica Judisch, the commissioning editor, was so very helpful in guiding a novice through the early phases of this book. Susan Engelken, the managing editor for this book, was incredible to work with, always patient, always competently and quickly responding to any issue or concern. Dr Joe Phillips, the copy editor Wiley enlisted, deserves the credit for identifying errors that I missed and enhancing the readability of this text.

Additionally, I would like to acknowledge on a personal level those who have been so important to me from the standpoint of my life and career. First of all I would thank my parents, Lewis and Barbara Hopper, who were always supportive of my goals and aspirations, and my family, wife Donna and children Tricia (her husband Caleb), McRae, and Molly, who I will always consider to be my greatest accomplishments. Also, as this goes to press I can announce a wonderful addition, a granddaughter by the name of Abigail Betty Butts.

I would also be remiss to not use this opportunity to thank some of my professors and instructors at Auburn who were so influential to me professionally and important to me personally. First on this list would be Dr Robert Hudson, but also Drs Bob Carson, Howard Jones, Donald Walker, Ram Purohit, John Winkler, and Howard Kjar. Likewise, I need to thank my colleagues at Mississippi State who have both encouraged and tolerated me through this long process.

I sincerely hope the reader finds this text useful.

Starkville, Mississippi *Richard M. Hopper*

About the Companion Website

The book is accompanied by a companion website at

www.wiley.com/go/hopper/bovine



The website includes:

- Figures in PPT
- Tables in PDF

Section I

The Bull

Anatomy and Physiology

1

Anatomy of the Reproductive System of the Bull

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Introduction

The anatomy of the reproductive system of the bull can be grouped functionally into the components of production, transport, and transfer of spermatozoa (Figure 1.1).

Production

The testicular parenchyma contains the cellular machinery for spermatogenesis and steroid production (Figures 1.2 and 1.3). The parenchyma is arranged in indistinct lobules of convoluted tubules called seminiferous tubules. The seminiferous tubules contain the spermatogonia from which the mature sperm cells develop. Sertoli cells are also located within the lumen of the seminiferous tubules. The Leydig cells that are responsible for the production of the male hormone testosterone are located between the seminiferous tubules in the interstitial space [1].

Testes

The testes are housed in the scrotum. The scrotum is suspended between the thighs in the inguinal region. The scrotum consists of external and internal layers. The external layer is made up of the skin, tunica dartos, superficial perineal fascia, external spermatic fascia, cremasteric fascia, internal spermatic fascia, and parietal vaginal tunic. The skin of the scrotum and tunica dartos muscle are closely adhered whereas the fascial layers easily separate from the skin and the parietal vaginal tunic as in a closed castration technique. The coverings of the testicle itself consist of the visceral vaginal tunic and the tunica albuginea [2]. The visceral vaginal tunic is the innermost layer of the vaginal tunic, an outpouching of abdominal peritoneum that passes through the inguinal canal into the scrotal sac. The potential space between the parietal and visceral vaginal tunic is the vaginal cavity (Figure 1.3). The purpose of the vaginal cavity is for temperature regulation of the testicle by raising it closer to the body through contraction of the tunica dartos and cremaster muscles. The tunica albuginea is a thick fibrous capsule that covers the testicle and maintains the testicular contents under pressure [3]. Internally the tunica albuginea forms the axially positioned mediastinum testis from which connective tissue septa divide the testis into indistinct lobules. This connective tissue framework supports the vasculature, nerves, parenchyma, and tubular system of the testicle. The scrotum of the bull is pendulous due to the dorsoventral orientation of the testes contained within [1].

Spermatic Cord

The spermatic cord includes the ductus deferens, vasculature, lymphatic vessels, and nerves of the testicle and epididymis [4]. Essentially the spermatic cord consists of all the tissue within the vaginal tunic so it extends from the vaginal ring within the abdominal cavity to the testicle [5].

Transport

Spermatozoa are transported from the testicles through a tubular system consisting of the convoluted seminiferous tubules, straight seminiferous tubules, rete testis, efferent ductules, epididymis, ductus deferens, and urethra (Figure 1.4). The tubular system allows for maturation and storage of spermatozoa and provides fluid to ease movement of the spermatozoa.

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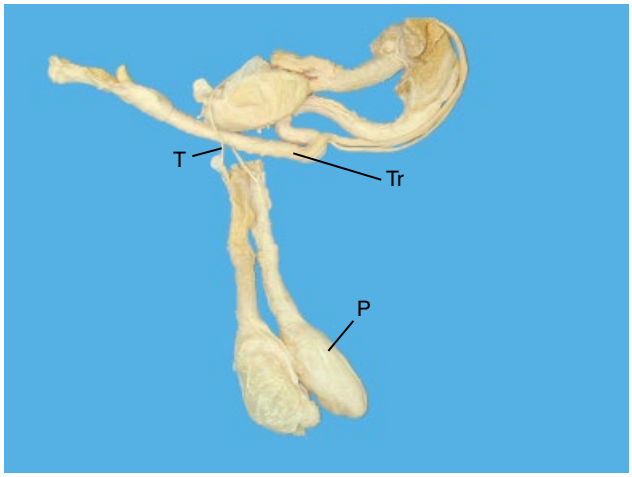


Figure 1.1 Components of spermatozoa: P = production, T = transport, Tr = transfer.

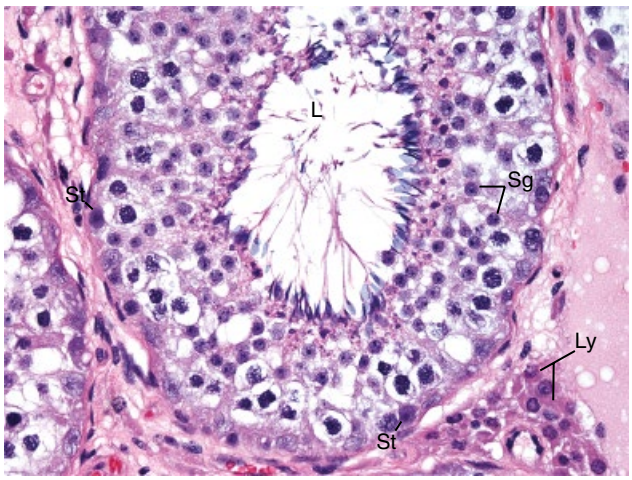


Figure 1.2 Histology of testicle: St = Sertoli cell, Ly = Leydig cells, Sg = spermatogonia L = lumen of seminiferous tubule.

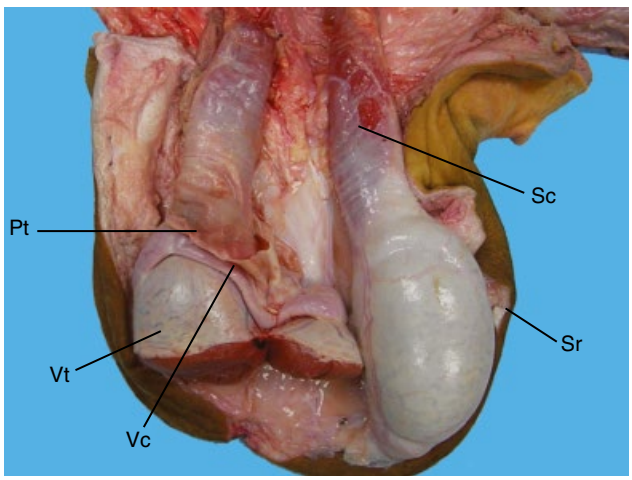


Figure 1.3 Tissue layers of testicle: Vt = visceral vaginal tunic, Pt = parietal vaginal tunic, Vc = vaginal cavity, Sc = spermatic cord, Sr = scrotum.

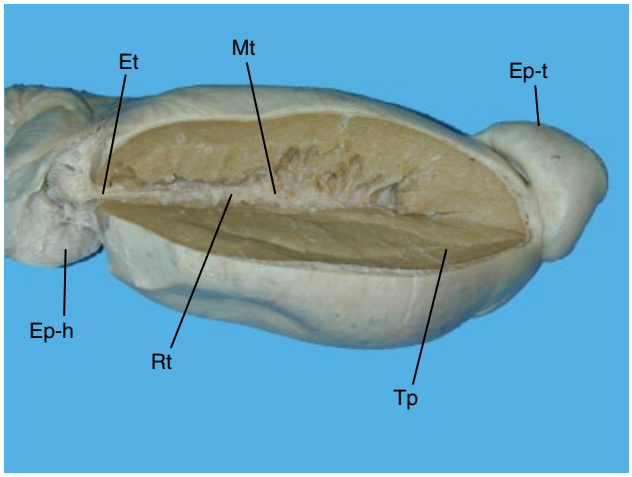


Figure 1.4 Ductules of testicle: Ep-h = head of epididymis, Et = efferent tubules, Mt = mediastinum testis, Rt = rete testis, Tp = testicular parenchyma, Ep-t = tail of epididymis.

Tubular Transport System

The convoluted seminiferous tubules are the location of the spermatogenic process: the development of spermatogonia to primary spermatocytes, to spermatids, and finally to spermatozoa [1]. This process occurs within the wall of the seminiferous tubule. Specific regions of the tubule are devoted to a particular stage of development, so that each stage can be identified by specific histological techniques [6]. Upon completion of spermiogenesis, the spermatozoa are released into the lumen of the convoluted seminiferous tubule to begin transit through the straight seminiferous tubule. The straight seminiferous tubule is simply the connection between the convoluted seminiferous tubule and the rete testis. The rete testis is a "network of irregular labyrinth spaces and interconnected tubules" [2]. The rete testes are located within the mediastinum testis connecting the seminiferous tubules to the efferent ducts that exit the testicle at the extremitas capitata (head). The efferent tubular system continues as the epididymis on the external surface of the testis (Figure 1.5). The epididymis is divided into a head, a body located on the medial surface, and a tail located at the distal extremitas caudate.

Ductus Deferens

The ductus deferens is attached to the medial side of the testicle by the mesoductus [5]. The ductus deferens is the continuation of the tail of the epididymis (Figure 1.6). The ductus deferens enters the abdominal cavity through the inguinal canal, crosses the lateral ligament of the bladder, and before ending at the colliculus seminalis in the urethra widens into the ampulla [5].

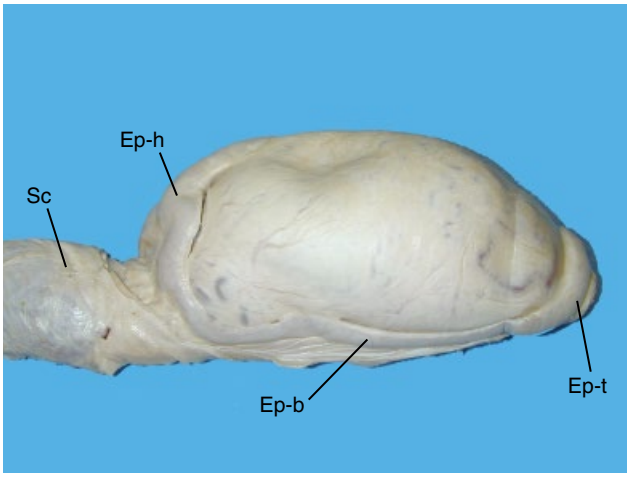


Figure 1.5 Components of epididymis: Sc = spermatic cord, Ep-h = head of epididymis, Ep-b = body of epididymis, Ep-t = tail of epididymis.

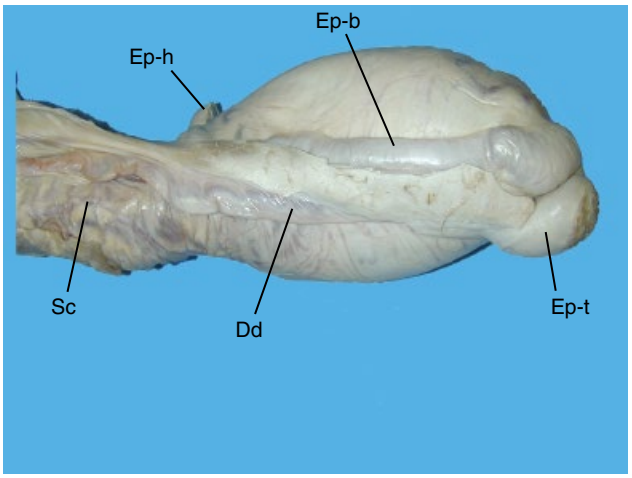


Figure 1.6 Components of epididymis: Sc = spermatic cord, Ep-h = head of epididymis, Ep-b = body of epididymis, Ep-t = tail of epididymis, Dd = ductus deferens.

Transfer

The transfer of spermatozoa from the bull to the cow is achieved by the process of intromission, which requires erection of the penis and ejaculation of sperm. The pertinent anatomy for these processes to occur includes the penis, the musculature of the penis, the vasculature, and the innervations.

Penis

The penis of the bull can be divided into a root, body, and glans penis (Figure 1.7). The root of the penis can be defined as the origin of the erectile tissue that comprises

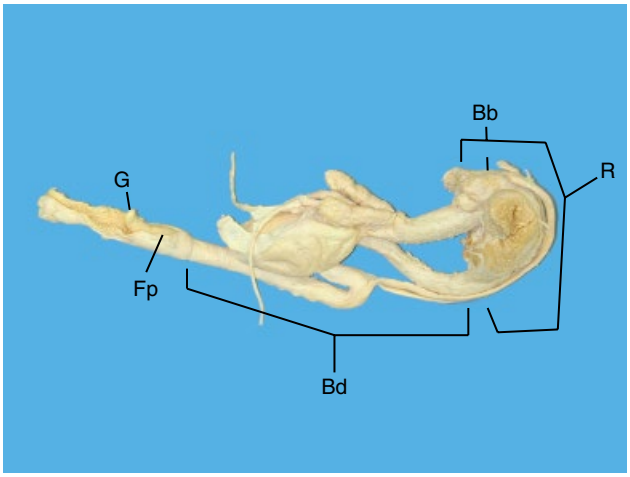


Figure 1.7 Regions of the penis: R = root of penis, Bb = bulb of penis, Bd = body of penis, Fp = free part of penis, G = glans penis.

the penis as well as the origin of the muscles of the penis. The erectile tissue that makes up the bulk of the penis is the corpus cavernosum. The paired corpora cavernosa originate separately on each side of the ischiatic arch medial to the ischiatic tuberosity. These individual limbs are termed the crura of the penis. The crura pass ventromedially until they join to form the body of the penis. The corpus spongiosum is the erectile tissue that surrounds the urethra. The origin of the corpus spongiosum, called the bulb of the penis, originates between the crura along the midline of the ischiatic arch. Therefore the root of the penis is composed of the crura (corpus cavernosum) and the bulb (corpus spongiosum).

The erectile tissue is enclosed in the dense outer covering of the tunica albuginea. The tunica albuginea is a dense covering that consists of an inner circular layer and outer longitudinal layer of fibers. The inner circular layer sends trabecular scaffolds throughout the corpus cavernosum for the attachment of the cavernous endothelium.

Located caudal to the root of the penis are the muscles of the penis: the ischiocavernosus, bulbospongiosus, and retractor penis muscles (Figure 1.8). The paired ischiocavernosus muscles originate on the medial surfaces of the ischiatic tuberosities overlying the crura; the muscle fibers pass ventromedially in a "V" fashion until ending a short distance on the body of the penis [1]. During erection the ischiocavernosus muscle contracts, pushing blood from the cavernous spaces of the crura into the body of the penis [7]. The bulbospongiosus muscle lies caudal to the bulb of the penis, originating along the ischiatic arch and continuing until the junction of the crura [1]. The bulbospongiosus muscle fibers run transversely across the bulb of the penis and contraction of this muscle results in propulsion of the ejaculate through the urethra [7]. The retractor penis muscle

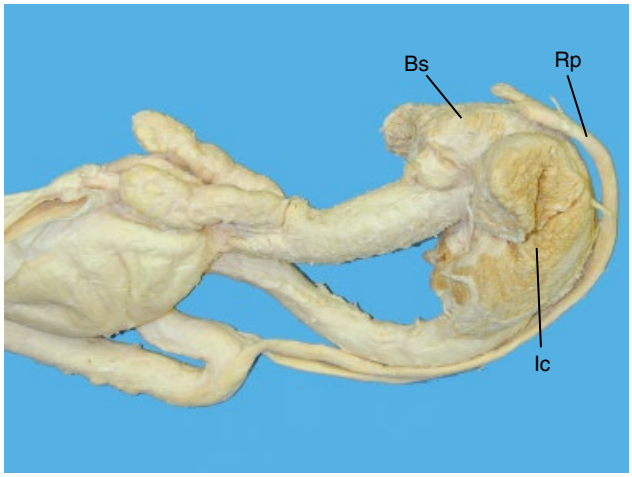


Figure 1.8 Muscles of the penis: Bs = bulbospongiosus muscle, Ic = ischiocavernosus muscle, Rp = retractor penis muscle.

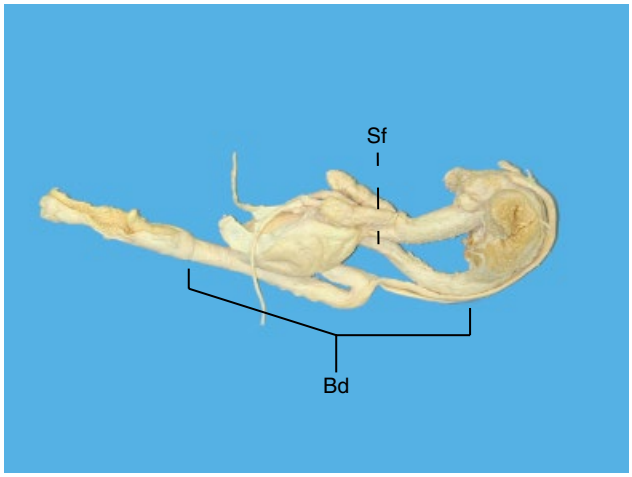


Figure 1.9 Sigmoid flexure of the penis: Bd = body of penis, Sf = sigmoid flexure.

extends from the caudal vertebrae and internal anal sphincter to insert distal to the sigmoid flexure [8]. These paired muscles relax during erection, allowing the penis to extend from the prepuce and contract during quiescence, retracting the penis into the sheath [8].

The body of the penis begins where the two crura meet distally to the ischiatic arch; it extends craniad, along the ventral body wall to become at the mid-ventral abdomen the free part of the penis (Figure 1.9). The body of the penis is bent in an "S" shape called the sigmoid flexure. The proximal bend of the sigmoid flexure opens caudally and is located near the scrotum. The distal bend is opened cranially and the short suspensory ligaments of the penis attach the penis to the ventral surface of the ischiatic arch. The glans penis is a small restricted region at the tip of the free part of the penis [8] (Figure 1.10). The free part of the penis is the distal extent from the attachment of the internal lamina of the prepuce to the glans penis [8]. The free end of the penis is twisted in a counterclockwise direction as viewed from the right side, illustrated by the oblique direction of the raphe of prepuce continued as the raphe of the penis to the urethral process (Figure 1.11). The twist of the free end of the penis is due to the attachment of the apical ligament. The apical ligament of the penis is formed by the longitudinal fibers of the tunica albuginea leaving the body of the penis just distal to the sigmoid flexure and reattaching near the apex of the penis [9].

The prepuce of the penis is composed of an external and internal fold or lamina [8] (Figure 1.12). The external lamina is the haired outer fold of skin attached to the ventral abdomen. The haired skin terminates at the preputial orifice where the external fold turns inward to line the preputial cavity as the internal lamina. The internal lamina serves to attach the external lamina to the penile epithelium.

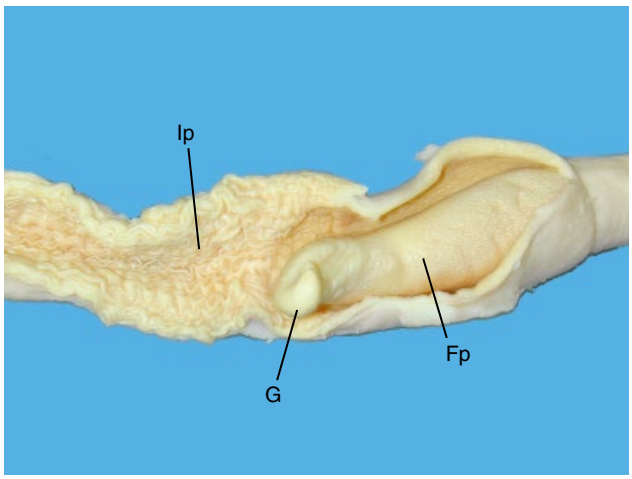


Figure 1.10 Glans penis and associated structures: G = glans penis, Fp = free part, Ip = internal lamina of prepuce.

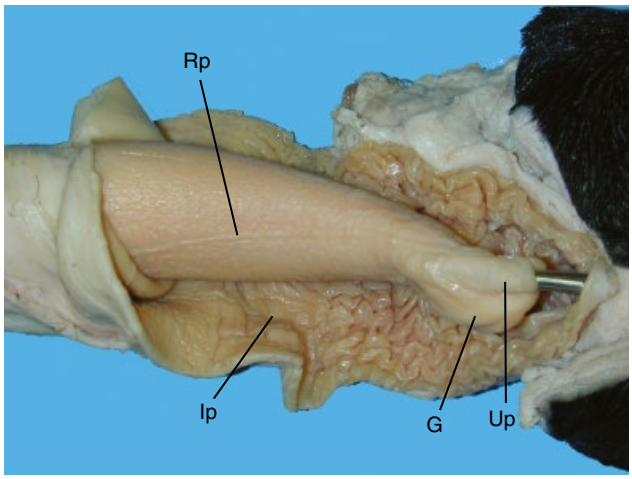


Figure 1.11 Prepuce and associated structures: Rp = raphe of penis, Ip = internal lamina of prepuce, G = glans penis, Up = urethral process.

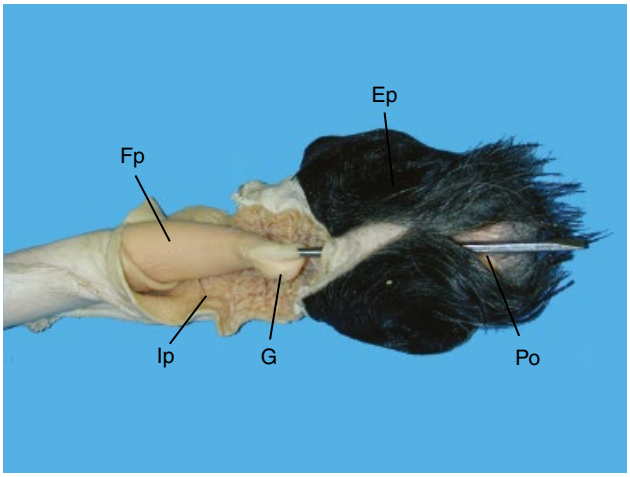


Figure 1.12 Components of the prepuce: Fp = free part of penis, G = glans penis, Ip = internal lamina of prepuce, Ep = external lamina of prepuce, Po = preputial orifice.

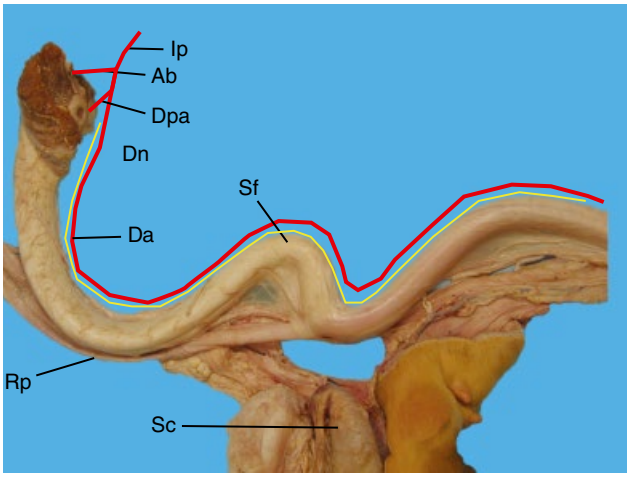


Figure 1.13 Arterial supply to the penis: Ip = internal pudendal artery, Ab = artery of the bulb of the penis, Dpa = deep artery of the penis, Dn = dorsal nerve, Da = dorsal artery of the penis, Rp = retractor penis muscle, Sc = spermatic cord, Sf = sigmoid flexure.

Blood Supply

Before ejaculation can occur the testis must produce spermatozoa. This requires an adequate blood supply for the metabolic demands of cellular division for spermatogenesis and steroidogenesis. The arterial blood supply to each testis is provided by a testicular artery, a direct branch of the abdominal aorta arising caudal to the renal arteries. The testicular artery crosses the lateral abdominal wall and then passes ventrally through the inguinal canal [10]. As the testicular artery approaches the testis it begins to spiral, with the nearby tortuous pampiniform plexus of the testicular vein forming a vascular cone. This arterial/venous arrangement is an effective thermoregulatory apparatus [11].

An adequate blood supply to the penis and associated muscles is required for the processes of erection, ejaculation, and tissue maintenance. This comes by way of the internal iliac artery. The internal iliac artery is a direct continuation of the abdominal aorta at the entrance to the pelvic cavity. The umbilical artery, a branch of the internal iliac, supplies the ductus deferens and the bladder [4]. The prostatic artery leaves the internal iliac and supplies the prostate, vesicular glands, ductus deferens, ureter, and urethra [4]. As the internal iliac continues through the pelvic cavity it divides into the caudal gluteal and internal pudendal [10]. The internal pudendal gives off the ventral perineal artery and urethralis artery, and continues as the artery of the penis [10]. The artery of the penis gives off the artery of the bulb of the penis, which supplies the bulbospongiosus muscle and the cavernous spaces of the corpus spongiosum [12] (Figure 1.13). The deep artery of the penis is another branch of the artery of the penis that enters the crus of the penis and supplies the erectile tissue, the corpus cavernosum [12]. After the deep artery branches off, the artery of the penis continues as the dorsal artery of the penis which passes along the dorsal aspect of the penis toward the glans penis and prepuce. It is responsible for maintenance of penile tissue during quiescence [13].

Nervous Supply

The innervation of the external genitalia of the bull consists of the pudendal nerve and its branches. The pudendal nerve carries motor, sensory, and parasympathetic nerve fibers [4]. The pudendal nerve passes through the pelvic cavity medial to the sacrosciatic ligament and divides as it approaches the lesser ischiatic notch of the pelvis into proximal and distal cutaneous branches supplying the skin of the caudal hip and thigh [4, 8]. The pudendal nerve continues through the ischiorectal fossa, terminating in a preputial branch, a scrotal branch, and finally the dorsal nerve of the penis [6]. The pelvic nerve provides parasympathetic innervations from the sacral plexus [1]. The hypogastric nerve contributes sympathetic fibers from the caudal mesenteric plexus to the genital system [1] (Figure 1.14).

Accessory Glands

The accessory genital glands of the bull include the vesicular gland, ampulla of the ductus deferens, and prostate and bulbourethral glands (Figure 1.15). The bilateral vesicular gland is the largest accessory gland in the bull and contributes the greatest volume to the ejaculate. It is a lobated gland of firm consistency. It lies dorsal to the bladder and lateral to the ureter and ampulla of the ductus deferens [1]. The body of the prostate lies dorsal to the

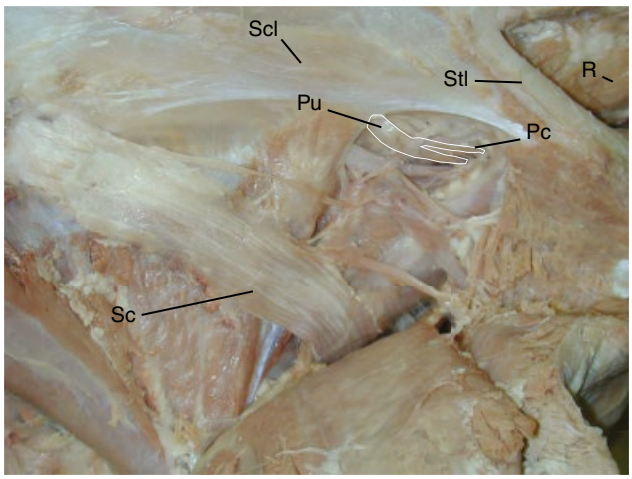


Figure 1.14 Nerves of the penis: Sc = sciatic nerve, Pu = pudendal nerve, Pc = proximal cutaneous branch of pudendal nerve Scl = sacrosciatic ligament, Stl = sacrotuberous ligament, R = rectum.

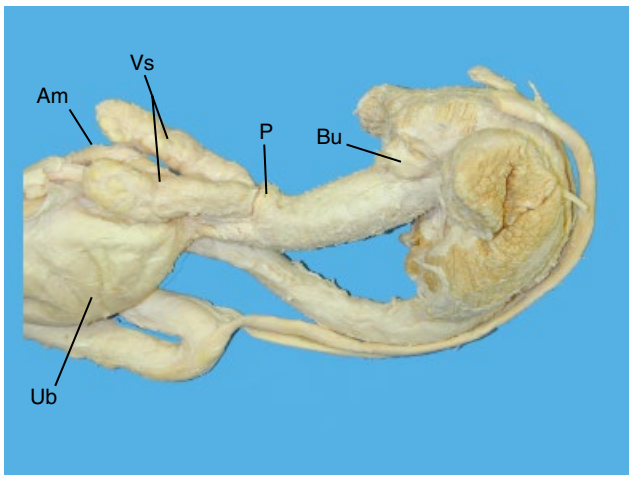


Figure 1.15 Secondary sex glands: Am = ampulla, Vs = vesicular glands, P = prostate, Bu = bulbourethral gland, Ub = urinary bladder.

urethra between and caudal to the vesicular glands. The disseminate part of the prostate is concealed in the wall of the urethra and covered by the urethral muscle [1]. The ampulla, vesicular glands, and prostate all empty their contents into the urethra through the colliculus seminalis. The bilateral bulbourethral gland lies on each side of the median plane dorsal to the urethra; it is mostly covered by the bulbospongiosus muscle. Its duct opens into the urethral recess [1] (Figure 1.15). The urethral recess is a blind pouch that exits dorsally into the penile urethra at the level of the ischiatic arch. The presence of this structure makes it difficult to pass a catheter retrograde into the bladder.

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2

Endocrine and Exocrine Function of the Bovine Testes

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Introduction

The normal bovine male reproductive system consists of paired testes retained within a sac or purse-like structure known as the scrotum, which is formed from the outpouching of skin from the abdomen and consists of complex layers of tissue. The testes are accompanied by a number of supporting structures including spermatic cords, accessory sex glands (prostate, bulbourethral, paired vesicular glands), penis, prepuce, and the male ductal system. The testicular duct system is extensive and comprises the vas efferentia found within the testes, epididymis, vas deferens, and urethra, all of which are located external to the testes. The reader is referred to the chapter on the anatomy of the reproductive system of the bull in this book (Chapter 1). The primary functions of the testes are to produce male gametes (spermatozoa) and the endocrine factors, such as steroid (testosterone) and protein hormones (inhibin, insulin-like peptide 3), which help regulate the reproductive function of the bull in concert with hormonal secretions from the hypothalamus (gonadotropin-releasing hormone) and pituitary glands (luteinizing hormone (LH), follicle-stimulating hormone (FSH)). The testes consist of parenchymal tissue that supports the interstitial tissue and includes the steroid-producing Leydig cells, vascular and lymphatic system, and seminiferous tubules within which the germinal tissue develops with the support of the nurse cells more commonly known as Sertoli cells. Chapter 5 discusses in detail the endocrine factors responsible for testicular development and initiation of spermatogenesis in the bull, and thus this chapter focuses more on the regulation and function of the adult testes. This chapter will not undertake a treatise of those conditions that disrupt testicular function but rather will focus, as practically as is possible, on what is known of the endocrine and exocrine function of the bovine testes. Much of the endocrine and exocrine function of the testes is similar across mammalian species, and where specific information is absent for the bovine, examples will be given from other domestic species when possible. It has not been possible to cite the many significant contributions to the field of endocrine and exocrine function of the testes. Thus, where and when possible, the reader is referred to selected citations for additional reading.

Historical Perspective

It has been evident for many centuries that the testes exercise control over the characteristics of the male body. The results of castration in domestic animals and human males made this very clear, but provided no clues as to the mechanism of control. Pritchard [1] noted from Assyrian records dating some 15 centuries BCE that the castration of men was used as punishment for sexual offenders, which suggests that the effect of castration on fertility and behavior was recognized at that time. Knowledge of the effects of castration of livestock dates back to the Neolithic Age (c. 7000 BCE) when animals were first thought to have been domesticated [2]. The effects of castration were understood by Aristotle (~300 BCE) who provided very detailed and clear descriptions of testicular anatomy and function [3]. It was not until the seventeenth century that a detailed account of testicular and penile anatomy was presented by Regnier de Graaf [4] in a treatise on the male reproductive organs. De Graaf indicated the existence of the seminiferous tubules and suggested that the production of the fertile portion of the semen occurred in the testes. The first microscopic examination of the testes was undertaken by Antonie van

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Leeuwenhoek in 1667 where he demonstrated and reported the presence of germ cells in the seminal fluid [5].

Detailed study of the testis began in the mid-nineteenth century. In 1840, Albert von Köllicker discovered that spermatozoa develop from cells residing in the testicular (seminiferous) tubules. This major discovery was followed by Franz Leydig's [6] description of the microscopic characteristics of the interstitial cells. Later, Enrico Sertoli [7], an Italian scientist, correctly described the columnar cells running from the basement membrane to the lumen of the *tubuli seminiferi contorti* (seminiferous tubules) of the testes, and Anton von Ebner is credited with introducing the concept of the symbiotic relationship between Sertoli cells and the developing germinal cells [8, 9].

The first clear demonstration that the testes are involved in an endocrine role was made by Arnold Berthold in 1849, while studying the testes of the rooster. He concluded that the regulation of male characteristics was brought about by way of blood-borne factors. The most compelling evidence for an endocrine function of the testes being associated with Leydig cells was presented by two French scientists, Bouin and Ancel in 1903. They reported that ligation of the vas deferens in dogs, rabbits, and guinea pigs was followed by degeneration of the seminiferous tubules, but no castration effects were observed and no degenerative changes of the interstitial cells, and thus it was concluded that internal secretions of the testes were synthesized by the Leydig cells [10]. By the mid-1930s it was clear that the male hormone emanating from the testes was testosterone [11] and that the function of the testes was controlled by pituitary hormones [12, 13]. Smith [12] demonstrated that the pituitary gland must secrete substances (now known as gonadotropins) responsible for the stimulation of testicular growth and maintenance of function in the rat. Greep et al. [14] restored Leydig cell function in hypophysectomized rats with crude preparations of LH and reestablished the male secondary sex characteristics. Further evidence was elucidated in favor of a steroid secretory function for Leydig cells from a study on postnatal development in bulls where changes in testicular androgen levels paralleled the differentiation of these cells [15]. Using cell culture techniques, Steinberger et al. [16] provided direct evidence that the Leydig cells are the primary source of steroid hormone synthesis in the testes. Later, it became apparent that the Leydig cells are essential for providing the androgenic stimuli that are required for the maintenance of spermatogenesis in the germinal epithelium [16, 17].

The Testis

The bovine testes are paired, capsulated, ovoid-like structures located in the inguinal region and suspended in a pendulous scrotum away from the abdominal wall. The proximal relationship of the testes to the abdominal wall varies and may depend on season and ambient temperatures. The cremaster muscle plays an important role in thermal regulation of the testis. The size of the testis varies with breed, but typically the adult testis weighs 300–400 g and is about 10–13 cm long and 5–6.5 cm wide [18]. The tough fibrous capsule covering each testis consists of three tissue layers: the outer layer, the tunica vaginalis; the tunica albuginea, which consists of connective tissue composed of fibroblasts and collagen bundles; and the inner layer, the tunica vaginalis, which supports the vascular and lymphatic systems [19]. The capsule is the main structure that supports the testicular parenchyma, the functional layer of the testes, which consists of the interstitial tissue and seminiferous tubules. The interstitial tissue is found in the spaces between the seminiferous tubules and consists of clusters of Leydig cells, which are primarily responsible for steroid hormone biosynthesis and secretion, along with vascular and lymph vessels that supply the testicular parenchyma. The seminiferous tubules originate from the primary sex cords and contain the germinal tissue (spermatogonia, the male germ cell) and a population of specialized cells, the Sertoli cells, which not only support the production of spermatozoa but also form tight junctions with each other, creating one of the most important components of the blood–testis barrier [20]. This structure prevents the entry of most large molecules and foreign material into the seminiferous tubules that may disrupt normal spermatogenesis. The most important substances synthesized by the testes and released into the vascular system are peptide and steroid hormones. However, fluids from the seminiferous tubules may pass into the interstitial tissue via the basal lamina, where they may enter the testicular lymphatic and vascular systems, or into the tubule lumen via the apical surface of the Sertoli cells [19].

The Scrotum and Spermatic Cords

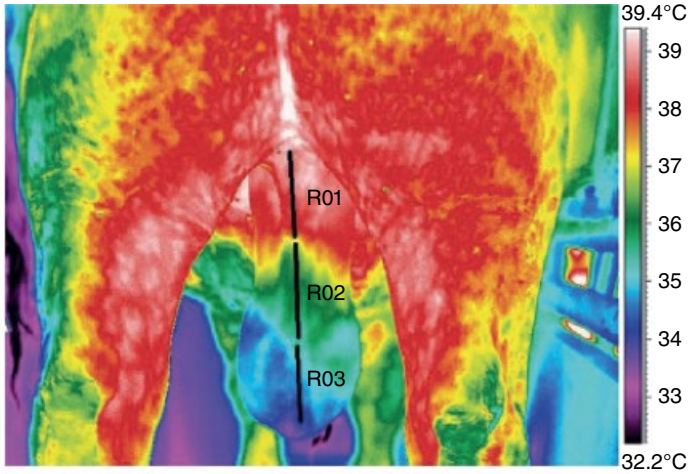
The scrotum is composed of an outer layer of thick skin and three underlying layers, the tunica dartos, the scrotal fascia, and the parietal vaginal tunica. The scrotal skin is extensively populated with numerous large adrenergic sweat and sebaceous glands that are highly endowed with thermal receptors and nerve fibers. Neural stimulation from the thermal receptors enables the tunica dartos, which consists of smooth muscle fibers and lies just beneath the scrotal skin, to contract and relax in response to changes in temperature gradients and facilitates the cooling of the scrotal surface via scrotal glandular sweating [19]. Thus the scrotum not only plays an important role in housing and protecting the testes but also has a role

in thermoregulation of the testes. The spermatic cord connects the testes to the body and provides access to and from the body cavity for the vascular, neural, and lymphatic systems that support the testes. In addition, the spermatic cord accommodates the cremaster muscle, the primary muscle supporting the testes, and the pampiniform plexus, a complex and specialized venous network that wraps around the convoluted testicular artery [21]. This vascular arrangement is very important in temperature regulation of the testicular environment. The plexus consists of a coil of testicular veins that provide a countercurrent temperature exchange system: this is an effective mechanism whereby warm arterial blood entering the testes from the abdomen is cooled by the venous blood leaving the testes. Testicular arteries originate from the abdominal aorta and elongate as the testis migrates into the scrotum [19]. In cattle and other large domestic ruminants these arteries are highly coiled, reducing several meters of vessel into as little as 10 cm of spermatic cord [19]. The arterial coils and venous plexus are complex structures that form during fetal life in cattle [19, 22]. Because of the pendulous nature of the bovine scrotum, testicular cooling is facilitated by the contraction and relaxation of the cremaster muscle, which draws the testes closer to the abdominal wall during cooler ambient temperatures and vice versa during warmer temperatures. Figure 2.1 shows bright-field and thermal images of the bovine testes that demonstrate the change in temperature from the neck to the tip of the scrotum as the testes thermoregulate during elevated environmental temperatures.

(a)



(b)



(c)

Temperature [°C]	Min	Max	Max–Min	Avg	Stdev
R01	37.7	39.1	1.4	38.6	0.3
R02	35.5	36.9	1.4	36.1	0.3
R03	34.0	35.5	1.5	34.7	0.4

Figure 2.1 Bright-field and thermal images of two-year-old Hereford bull testes. The images were taken using an FLIR SC600 thermography unit (FLIR Systems Sweden, AB) on 25 June 2013 at midday with atmospheric temperature of 29 °C and humidity of 75%. (a) Bright-field image of the testes. (b) Thermal image of the testes as seen in (a), identifying three regions of interest (RO1, RO2, and RO3) as indicated by the vertical bars along which temperature values were obtained by means of the software ThermaCAM Research Pro 2.7. The image is pseudo-colorized with temperature scale bar to help visualize the change in temperature gradient from scrotal neck to scrotal tip. (c) Temperature values, minimum (Min), maximum (Max), the difference between Min and Max, average (Avg) temperature for each region of interest (RO), and standard deviation (St dev) which assess the variation of temperature in each region. Note the almost 4 °C change in average temperature from RO1 to RO3.

Scrotal and testicular thermoregulation is a complex process involving a number of local mechanisms that strive to maintain the testes at environmental and physiological conditions conducive for normal spermatogenesis. For additional reading on testicular thermoregulation in the bull the reader is referred to the review by Kastelic et al. [23] and Chapter 4 of this book.

Interstitial Tissue (Leydig Cells)

Franz Leydig, a German zoologist, first described the interstitial cells of the testes in 1850 and these cells have since been known as Leydig cells. The Leydig cells reside in the interstitial tissue of the testis, a meshwork of loose connective tissue filling the spaces between the seminiferous tubules and blood vessels. In mammalian testes, the Leydig cells occur mainly as clusters in the angular interstices between the seminiferous tubules and are closely associated with the walls of small arterioles [8, 24]. The Leydig cell content of testes varies from species to species. The Leydig cells are thought to be the principal source of androgens in the testis. The development of the Leydig cells, via metamorphosis of mesenchymal precursor cells, has been observed to be continuous throughout life after the time of puberty in the bull [25]. Christensen [26] provides a very detailed and interesting review of the history of Leydig cell research dating from Leydig's description of the cells in the 1850s to the confirmation provided in the mid-1960s that these cells were indeed primarily responsible for testicular androgen synthesis and secretion. There is extensive evidence to suggest that early fetal Leydig cells are steroidogenically active in some mammalian species including the pig [27] and sheep [28].

The Leydig cells of most mammalian species studied are basically similar, with some minor variations in appearance, size, and the relation of Leydig cell clusters to the lymph or blood vessels of the interstitial tissue. Some variation in the extent of cytoplasmic structures exists, but ultrastructurally Leydig cells show considerable overall similarity [8]. Fawcett et al. [29] described in detail the morphology of interstitial tissue of several mammalian species, and categorized three groups based on the abundance of Leydig cells and the relationship between volume of intertubular lymph structures and connective tissue. In the first group are the guinea-pig and rodents (rat, mouse). In these species only 1–5% of the testicular volume is occupied by Leydig cells, for example 2.8% in the rat [30]. The bull, monkey, elephant, and human fall into the second group. In these species, the connective tissue of the interstitium is very loose and the Leydig cells are scattered throughout the interstitium and are closely associated with a well-developed lymph system. The Leydig cells comprise only a small portion of the testicular volume (~15%) [8]. In the third group are the domestic boar and horse. In these animals there is abundant interstitial tissue packed with Leydig cells (20–60% of testicular volume) [31]. The reason for the high density of Leydig cells in these species is not known, but Parkes [10] and Fawcett et al. [29] have attributed this phenomenon to the vast amounts of estrogens produced by the boar and stallion and the large quantities of musk-smelling 16-androstenes secreted by the boar testes [32]. For more detailed discussions on the cytology of Leydig cells the reader is referred to an excellent chapter by de Kretser and Kerr [33].

Endocrine Function of the Testis

Hypothalamic–Pituitary Hormone Regulation of the Testis

Hormone action depends on the release of hormones from the appropriate endocrine gland and transportation via the vascular circulatory system to the target tissue where the hormone binds to cellular receptors, thus inducing a physiological response. In some cases these receptors are very hormone-specific. The response at the target tissue may depend on the level of receptor expression and concentration of hormone. Some hormones regulate their own receptors, others may require synergism between two hormones, and others still may have their receptors regulated by other hormones [34]. The general characteristics of neuroendocrine regulation of the mammalian testis by the hypothalamic–pituitary axis are well established [35], but in some species this may be seasonally regulated. Domestic cattle are considered to be continuous or non-seasonal breeding species [36]. However, there is information in the literature to indicate that domestic beef and dairy bulls have a functional hypothalamic–pituitary–gonadal axis that is seasonally regulated and thus may influence levels of gonadal steroidal and germ cell production. In a study using composite breeds of mature bulls, Stumpf et al. [37] observed that season of the year influenced the profile of gonadotropins in the circulation of both gonadectomized and intact males, and that the greatest secretion of gonadotropins occurred at the spring equinox. In addition, these authors observed that season of the year also influenced secretion of testosterone in intact males, but that more testosterone was released in response to LH during the time of the summer solstice. Others have reported similar effects, where location affected reproductive traits of bulls including semen quality and blood concentrations of LH and testosterone [38, 39]. In addition,

sensitivity in responsiveness to exogenous gonadotropin administration and testosterone secretion in bulls was observed to be seasonally influenced [40].

The regulation of testicular function by hormonal mechanisms depends on the integrated actions of gonadotropins, such as LH, FSH, and prolactin, and steroids (androgens and estrogens) on the Leydig cell [41]. Gonadotropin-releasing hormone is the primary hypothalamic hormone governing regulation of the synthesis and release of the gonadotropins LH and FSH by the anterior portion of the pituitary gland. LH is primarily responsible for testosterone production by the Leydig cells, while FSH facilitates Sertoli cell proliferation and support of the germinal cells. Although it has been well established that gonadotropins stimulate testicular function, it was during the 1970s that the basic mechanism and site of action in the testis were identified. LH has been shown to be the gonadotropin essential for the maintenance of testicular testosterone production [41, 42]. Catt and Dufau [43] have reviewed the mechanisms of action of the gonadotropins and concluded that they all elicit target cell response by similar mechanisms. In fact, LH provides the most important physiological regulation of the production of androgens by the Leydig cells of the testis [44, 45].

Leydig cells contain plasma membrane-bound receptors that specifically bind LH. The binding of LH stimulates adenyl cyclase to produce cyclic adenosine monophosphate (cyclic AMP), a second messenger in the cell cytoplasm, which in turn activates cyclic AMP-dependent protein kinase, thereby increasing the conversion of cholesterol to pregnenolone. The action of LH can be readily demonstrated in hypophysectomized and intact males. Removal of the pituitary gland is followed by rapid cessation of testosterone production, loss of enzymes involved in steroidogenesis, and testicular atrophy [46]. There is little direct evidence to support a role of FSH in Leydig cell steroidogenesis, but there is some evidence indicating that this gonadotropin may play a function in the conversion of androgens to estrogens in the Sertoli cells [47]. Bartke et al. [42] have suggested that FSH may act on the aromatizing enzyme system of testosterone biosynthesis, but little evidence exists for action on other steps of the steroidogenesis pathway in the testis. In bulls, testosterone secretion is not tonic, but is characterized by episodic pulses dictated by the release of luteinizing hormone releasing hormone (LHRH) from the hypothalamus and LH from the anterior pituitary gland [48]. Schanbacher [48] reports that a temporal relationship exists between concentrations of LH and testosterone in the blood, and that there is evidence that episodic secretion depends on discrete episodes of LHRH discharge from the hypothalamus.

Prolactin

Prolactin (PRL) is known to enhance the effect of LH on spermatogenesis. The physiological importance of PRL in the regulation of Leydig cell function was first determined using three animal models [41, 42]: the golden hamster, where testicular regression can be induced by short photoperiod; hypophysectomized mice; and the hereditary dwarf mouse with congenital PRL deficiency and infertility. In all three models there is a deficiency in plasma concentrations of PRL and testosterone and testicular atrophy is evident. PRL therapy, on the other hand, stimulates spermatogenesis in the dwarf mouse and restores testicular function in the golden hamster; in hypophysectomized animals, PRL, in the presence of LH, induces spermatogenesis and restores plasma testosterone. In men, PRL has an important role in the control of testosterone and reproductive function [49]. At very high concentrations (hyperprolactinemia), PRL has an antigonadal effect in men, inhibiting testicular function, and is associated with hypogonadism [50].

Other Regulatory Factors

The effects of other regulatory factors, such as growth hormone (GH), on testicular steroidogenesis have not been fully clarified. However, in view of the structural similarities between PRL and GH, it is thought that both hormones may have comparable effects on Leydig cell function [42]. The effect of GH may be more significant during puberty (see Chapter 6). However, administration of gonadal steroids to intact animals is known to cause reduced androgen production, an effect that has been attributed to inhibition of gonadotropin secretion with secondary effects on testicular endocrine function. Estrogen receptors have also been detected in interstitial tissue of the testis and since decreased testosterone levels are not correlated with a corresponding change in plasma LH, some authors suggest that estrogen may exert a direct inhibitory action on Leydig cell function [51, 52]. The effects of corticosteroids on Leydig cell function have been noted. Boars treated with adrenocorticotropin (ACTH) experienced increased testicular testosterone simultaneously with increased secretion of adrenal corticosteroids [53]. A transient increase in peripheral circulation of both corticosteroids and testosterone was first observed in boars following acute treatment with ACTH, while a decrease in testosterone occurred following chronic treatment [54]. These authors concluded that prolonged stressful conditions may lead to chronic elevation in ACTH levels, thus suppressing testosterone production and inducing poor breeding activity.

In reviewing the literature, Tilbrook et al. [55] have determined that stress-related influences on reproduction

are complicated and not fully understood, but the process may involve a number of endocrine, paracrine, and neural systems. The significance of stress-induced secretion of cortisol varies with species. In some instances, there appears to be little impact of short-term increases in cortisol concentrations, and protracted increases in plasma concentration seem to be required before any deleterious effect on reproduction are apparent. In a comprehensive review, Moberg [56] describes the influence of the adrenal axis on gonadal function in mammals. Subsequently, the same author published a detailed review [57] of the impact of behavior on domestic animal reproductive performance and placed particular emphasis on how intensive livestock management practices can contribute to stress-induced disruption of normal reproductive function. However, the emphasis has primarily been on studies investigating the effects of stress-induced ACTH release in females.

Elevated plasma ACTH results in increased adrenal corticosteroid synthesis and under chronic conditions may inhibit gonadotropin-releasing hormone and gonadotropin hormone production, thereby reducing gonadotropin (LH) release from the pituitary gland and thus impacting normal ovarian function in females. Nevertheless, studies have shown that stress-induced elevation in ACTH affects males in a physiologically similar manner by impacting gonadotropin regulation of testicular function. Liptrap and Raeside [58] observed the effects of cortisol on gonadotropin-releasing hormone in boars, while Matteri et al. [59] observed that stress or acute ACTH treatment suppresses gonadotropin-releasing hormone-induced LH release in the ram. In Holstein bulls treated with a bolus of ACTH (0.45 IU/kg), plasma LH or FSH concentrations did not appear to be negatively affected, but it did appear to reduce plasma testosterone concentrations [60]. Pharmacological concentrations of ACTH (200 IU every eight hours) over a six-day period resulted in reduced plasma testosterone in yearling and mature bulls within eight hours and four days of initial treatment, respectively, and this decrease persisted in both age groups for an additional 24 hours after the last ACTH injection [61]. While semen viability, concentration, and sperm output were unaffected by the prolonged ACTH treatment concomitant with a subsequent marked increase in glucocorticoids and decrease in testosterone, a small increase in semen content of immature sperm or sperm with abnormal heads was observed [61]. Thus, it appears that under prolonged stressful conditions, gonadotropin secretion is more likely to be suppressed, thus inhibiting reproductive performance. In addition, studies have demonstrated that glucocorticoids can inhibit gonadotropin secretion in some circumstances. Tilbrook et al. [55] conclude that suppression of reproduction is more likely to occur under conditions of chronic stress and may involve actions at the level of the hypothalamus or pituitary. In addition, they indicate that there are likely to be species differences in the effect of glucocorticoids on gonadotropin secretion, and the presence of sex steroids and the sex of an individual are also likely to be factors.

Cytokines are members of the family of growth factors, and the interleukins in particular may act at the level of the hypothalamus where they may control the release of gonadotropin-releasing hormone, thus influencing the release of pituitary gonadotropins and ultimately gonadal steroid synthesis and release. Svechniko et al. [62] have reported that testicular interleukin (IL)-1 may play a role in the paracrine regulation of Leydig cell steroidogenesis in rats. Others have reported that testicular interleukins may play an important functional role in both normal testicular function and under pathological conditions [63]. There is some evidence in the bovine that cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ may play a role in nitric oxide regulation in luteal endothelial cells by increasing inducible nitric oxide synthase (iNOS) activity, thereby accelerating luteolysis, while progesterone is thought to suppress iNOS expression in bovine luteal cells [64]. Whether these or other cytokines have similar effects on testicular cells of the bull has yet to be reported.

Steroid Synthesis by Leydig Cells

Since the beginning of the twentieth century, Leydig cells have been considered the probable source of testicular androgens [65]. Berthold [66] was the first to observe from experimentation with the rooster that the testes produced a substance that influenced secondary sex organ development and maintenance [26]. The first isolation of an androgen, androsterone, from human urine [67] and the crystallization of testosterone from bull testes [11] established the major site of testosterone production as the testis. Extensive literature has emerged over the past 50 years on the function of the Leydig cell. These studies, employing a variety of techniques, have identified and confirmed the Leydig cells as the primary source of testicular androgens and elucidated the important pathways in androgen biosynthesis [5, 26, 52, 68]. LH has also been confirmed as the major pituitary hormonal stimulus on the Leydig cells [46]. Ewing et al. [68] have suggested that because Leydig cells are concentrated in clusters in the interstitial tissue of the testis, they must influence seminiferous tubular and peripheral androgen-dependent functions (accessory sex glands) by hormonal signals rather than by cell-to-cell interaction.

The primary steroids produced and secreted by the bull testis are shown in Table 2.1. The conversion of the precursor cholesterol to androgens and estrogen is facilitated by a

Table 2.1 Primary steroid and peptide hormones synthesized and/or secreted by the bull testis.

Hormone family	Hormone	Site of synthesis
Steroid family		
Cholesterol (27 carbons)	Cholesterol 22-hydroxycholesterol 20,22,-dihydroxycholesterol	*De novo* biosynthesis, fat deposits, or from blood
Progestins (21 carbons)	Δ5-Pregnenolone 17α-Hydroxypregnenolone 17α-Hydroxyprogesterone Progesterone	Leydig cells (mitochondria)
Androgens (19 carbons)	Dehydroepiandrosterone Δ4-Androstenedione Δ5-Androstenediol Testosterone Dihydrostestosterone	Leydig cells (microsomal compartments)
Estrogens (18 carbons)	Estrone Estradiol-17β	Leydig cells
Peptide family		
Relaxin-like peptides	Relaxin/insulin-like peptide-3	Leydig cells
Neuropeptides	Oxytocin Glial cell-derived factor	Leydig cells Seroli cells
Cytokine family		
	Activin Inhibin	Sertoli cells Sertoli cells
Glycoproteins		
	Androgen binding protein Testicular transferrin	Sertoli cells Sertoli cells

series of enzymatic reactions involving hydroxylation, dehydrogenation, isomerization, C–C side-chain cleavage (lyase), and aromatase activity. Testosterone is now recognized as the principal steroid responsible for the endocrine functions of the testis since it is synthesized in copious amounts by mammalian Leydig cells. The Leydig cell has also been identified as a major source of other androgens and estrogens [5, 69]. In addition, the Leydig cells of the boar testis produce large amounts of the musk-smelling steroids, Δ^{16}-androstenes [70]. It has become apparent that the primary function of the Leydig cell is the provision of the androgen stimulus required for the initiation and maintenance of spermatogenesis in the germinal epithelium within the seminiferous tubules [71]. Some of the earliest functions of the Leydig cell are associated with regulation of the male reproductive organs, the organization of parts of the brain, pituitary secretion of gonadotropins, and accessory sex organ development of the neonate to ensure the appropriate response by these tissues to testicular steroids in the adult male [69]. The dependence of male sex accessory organs on testicular hormones is not restricted to the early fetal period but occurs also during puberty and throughout male adult life [72].

Cholesterol has been described as an obligatory intermediate in testosterone synthesis [5]. Testosterone is synthesized from a pool of metabolically active cholesterol, which is derived from either de novo biosynthesis of cholesterol, cholesterol esters stored as lipid droplets in the cell cytoplasm, or from blood plasma [68]. The conversion of cholesterol to testosterone involves five main enzymatic steps that include 20,22-lyase, 3β-dehydrogenase isomerase, 17α-hydroxylase, 17,20-lyase, and 17β-hydroxysteroid dehydrogenase, and in some species the conversion of androgens to estrogens via the aromatase enzyme system [73]. Conversion of cholesterol to pregnenolone is the initial step in the pathway and is catalyzed by the cholesterol side-chain cleavage enzyme complex, a three-step process that takes place in the mitochondria of the cell [45]. Briefly, pregnenolone is formed from

cholesterol (C27 sterol) by cleavage of the bond between C20 and C22 catalyzed by the multienzyme complex of the side-chain cleavage system in the mitochondria, and metabolized in the microsomes by the microsomal enzyme complex 3β-hydroxysteroid dehydrogenase/isomerase. Pregnenolone is also an obligatory intermediate in Leydig cell steroid synthesis. Van der Mollen and Rommerts [52] have indicated that the conversion of C21 steroids (i.e. pregnenolone) to C19 steroids (testosterone) may occur in the mammalian testis through two biosynthetic pathways. The crucial step in the biosynthesis of pregnenolone to androgens is the cleavage of the two-carbon side-chain of 17α-hydroxyprogesterone or 17α-hydroxypregnenolone by the 17,21-lyase enzyme complex [74]. This is regarded as an irreversible reaction producing "weak" androgens (androstenedione and dehydroepiandrosterone, respectively). Through the action of 17β-hydroxysteroid dehydrogenase, androstenedione is converted to the more potent androgen testosterone, which occurs in the microsomal compartments of the cell. However, testosterone is converted to dihydrotestosterone (DHT) by the 5α-reductase enzyme system, and is regarded as the more biologically active androgen produced by the testis. A more detailed account of the mechanisms involved in testosterone biosynthesis can be found in the review by Hall [45], but a schematic overview of steroid synthesis in the bovine testis is presented in Figure 2.2.

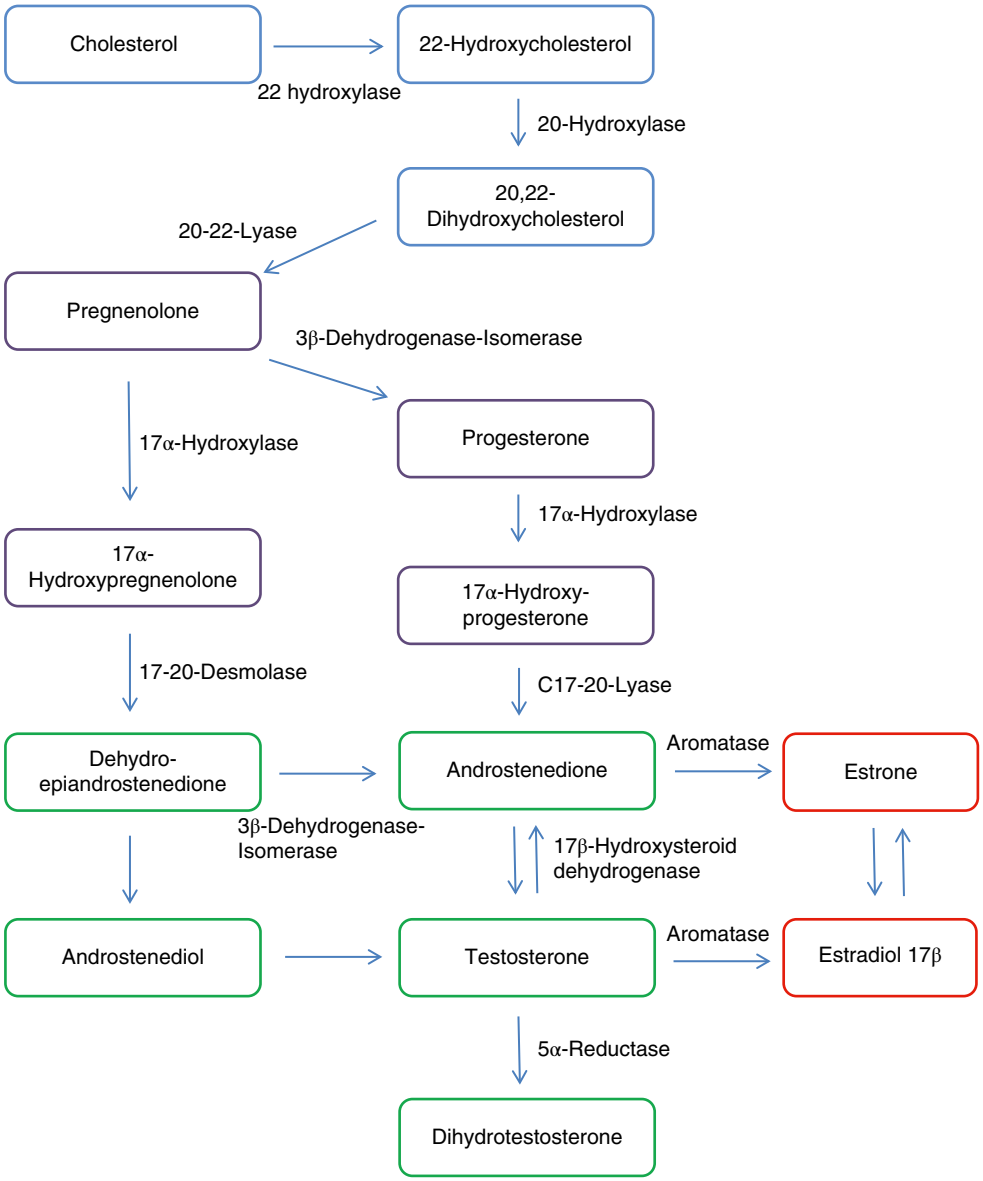


Figure 2.2 Synthetic pathway of testosterone and conversion to active androgen and estrogen metabolites in the bull testis. Relevant enzyme systems involved in the synthesis are shown; in some instances, the enzyme reactions are reversible. Color code: blue, C_{27} cholesterol steroid precursors; purple, C_{21} progestin steroids; green, C_{19} androgen steroids; red, C_{18} estrogen steroids.

The unusual abundance of Leydig cells in domestic boars and stallions promoted the hypothesis that this may be related to the fact that both species secrete significant amounts of estrogens [75], but the significance of the vast quantities of estrogens produced by these two species is unexplained. It has been suggested that estrogens act synergistically with testosterone to enhance both secretory activity of accessory sex organs and sexual behavior in boars castrated after puberty [76]. Estrogens are C18 steroids and are formed by the conversion of androgens by the aromatase enzyme system to produce estrone and estradiol from androstenedione and testosterone, respectively. Of interest in the boar are the musk-smelling Δ^{16}-androstene steroids (pheromones) that are regarded quantitatively as the most abundant steroids produced by the boar testis and contribute to the familiar "boar taint" of pork [77]. However, there is insufficient evidence to demonstrate that bull testis produces estrogens in the quantities found in the boar and stallion, nor is there evidence that the bull secretes much in the way of the Δ^{16}-androstene steroids. However, what is now well documented is that testosterone is the most potent androgen produced by Leydig cells in mammalian testes, and the site of action is primarily on seminiferous tubule target cells, thus influencing the reduction division of the spermatogenic cells [78]. Androgens stimulate production of androgen-binding protein (ABP) by the Sertoli cells [79], and this acts as an intracellular carrier of testosterone and DHT within the Sertoli cells. Testosterone is also the most important determinant of the rate of formation of fructose by the seminal vesicles and of citric acid by the prostate and seminal vesicle glands of the bull, ram, and human [78].

Oxytocin is a nine amino acid neuropeptide hormone normally associated with the hypothalamic–posterior pituitary system and the regulation of parturition and lactation in the female, but has also been shown to have an endocrine and paracrine role in male reproduction [19, 80]. There is evidence reported in the literature that oxytocin is produced and secreted by the male reproductive tract including the testis [81, 82]. Moreover, there is now evidence to show that oxytocin is produced locally by the testis and that it has a paracrine role in modulating testicular steroidogenesis and contractility of the male reproductive tract [83]. In addition, it has been shown that the Leydig cells are the testicular site of production of this hormone, and that oxytocin acts as a paracrine hormone influencing the contractility of the peritubular myoid cells [19]. The contraction of myoid cells in the seminiferous tubule epithelium is thought to facilitate sperm transport through the testicular parenchyma emptying into the rete testis and on into the epididymal system. It has been shown that, within the prostate, testosterone is converted by 5α-reductase to DHT, which stimulates growth of the prostate gland. Nicholson [83] has postulated that oxytocin increases the activity of 5α-reductase, resulting in increased concentrations of DHT and growth of the prostate, but that androgen feedback reduces oxytocin concentrations in the prostate, thereby modulating prostate gland growth. Definitive evidence of oxytocin synthesis within the bovine testis has come from studies on oxytocin gene expression in the seminiferous tubules [84, 85].

Relaxin and Insulin-like Peptide (INSL-3)

Increasing evidence emerging in the literature indicate that relaxin and relaxin-like peptides play an important role in male reproductive function. Insulin-like peptide 3 (INSL3; formerly known as relaxin-like factor 3) is a peptide hormone belonging to the relaxin–insulin family of peptide hormones [86–88] and binds to the RXFP2 receptor complex [89] to initiate physiological responses. It was first identified in the testes of pigs [90] and is now thought to be an important factor in regulating normal testicular decent [87], particularly during the second phase where it acts on the gubernaculum, as demonstrated using knockout mice. The peptide hormone has been identified in male and female tissues of other species, including human, marmoset monkey, sheep, goat, bovine, as well as deer, dog, and other species [88]. It is produced in large quantities by the Leydig cells of both the fetal and adult testes, and circulating INSL3 concentrations have been measured in the blood of adult male mammals including the rat (5 ng/ml) [91], mouse (2 ng/ml) [91], and human (0.8–2.5 ng/ml) [92, 93]. Although INSL3 has been successfully extracted from bovine testis [94] and found to be present in amniotic fluid from human male fetuses [95], it is only recently that Anand-Ivell et al. [96] using new time-resolved fluorescence immunoassay to directly detect INSL3 in the blood and body fluids of ruminants reported that mid-gestation (day 153) cows carrying a male fetus showed significantly higher maternal blood concentrations of INSL3 compared with cows carrying a female fetus. The authors speculate that INSL3 provides the first example of a gender-specific fetal hormone with the potential to influence both placental and maternal physiology. In a recent review, it is reported that INSL3 is a major secreted product of the interstitial Leydig cells of the mature testes in all mammalian males [97]. Moreover, current evidence points to autocrine, paracrine, and endocrine roles, acting through the G-protein coupled relaxin family receptor 2 (RXFP2), although more research is required to characterize these functions in detail. Indeed, recent studies have provided evidence of the presence of both RXFP1 and RXFP2 receptors in porcine spermatozoa [98], which would suggest that relaxin may play an important role in sperm production motility and [99]. Disruption of the INSL3 binding to its receptor complex RXFP2 demonstrated the importance of

this hormone in promoting and maintaining normal sperm production in the boar testis [100]. Pitia and colleagues [101] investigated the functional INSL3 hormone-receptor system in the testes and spermatozoa of several domestic ruminant species including bulls, rams, and goats, and explored the potential of this hormone-receptor to evaluate the fertility of sires. In all ruminants examined, the study identified the presence of RXFP2 in the Leydig cells, testicular germ cells, and sperm (equatorial segment) of fertile males, but in sub-fertile bulls, the expression pattern in these tissues was significantly reduced. Moreover, the study observed a significant reduction in spermatozoa INSL3 binding in semen recovered from sub-fertile bulls [101]. Consequently, the authors proposed that poor INSL3 binding by spermatozoa may have potential in predicting sire fertility.

Relaxin

The physiological role of relaxin has been intensively studied in many species over the years, including domestic ruminants, with most of the emphasis on the role in pregnancy. Experimental evidence suggests that a number of species in the Bovidae family, both domestic and non-domestic species, do synthesize relaxin, but apparently respond to it in a physiological manner [102–104]. Specific studies performed by Bagna et al. [102] and Musah et al. [105] explored the efficacy of purified porcine relaxin on parturition in beef and dairy cattle, indicating the possible presence of relaxin RXFP receptors in the bovid female. Observing the experimental evidence suggesting that cattle may not synthesize relaxin, but appear capable of responding to the hormone physiologically, Malone et al. [106] undertook an investigation to characterize the genomic locus of the relaxin gene (RLN1) in Laurasiatherian mammals to understand how cattle may have lost the ability to synthesize this peptide hormone. The outcome of these studies revealed that both domestic and wild ruminant species, including the cow, giraffe, Tibetan antelope, sheep, and goat, lack a functional RLN1 gene. Moreover, the study documented the progressive loss of RLN1 in the evolutionary lineage leading to cattle (Figure 2.3) and that the cow genome has lost all trace of the gene [106]. Interestingly, the same study confirmed that all bovids examined possess copies of the relaxin receptors, RXFP1 and RXFP2, which may explain why in some studies, dairy and beef cattle respond to relaxin despite the inability to synthesize the hormone [106].

The importance of relaxin is less well studied in male mammals. The presence of relaxin-binding activity has yet to be clearly demonstrated in the male bovid, but with the advent of new technologies this may be possible [107]. However, Kohsaka et al. [108, 109] have demonstrated, utilizing immunohistochemical studies, the binding of relaxin to spermatozoa and suggested that this activity may impart physiological effects on sperm motility and fertility.

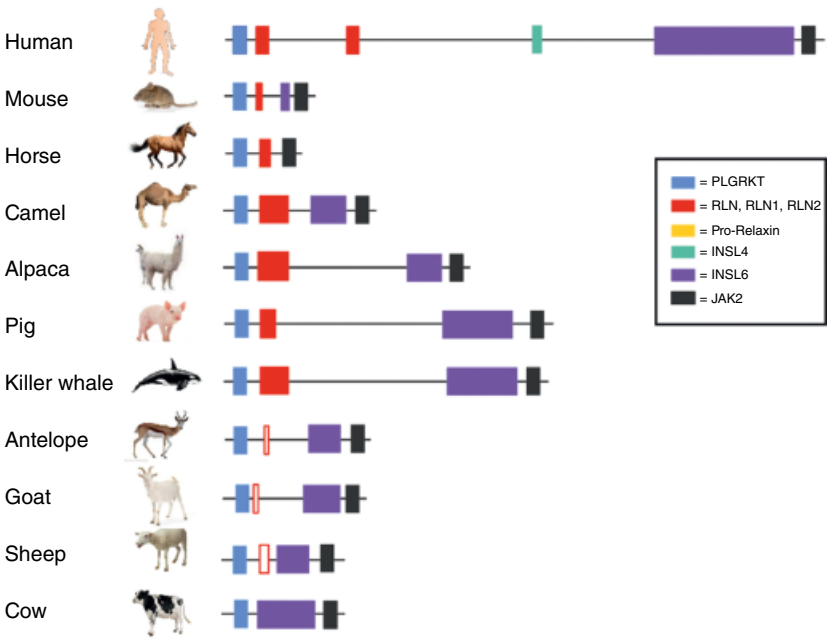


Figure 2.3 Genomic context of the relaxin gene in selected mammals. The cluster containing the RLN, INSL4, and INSL6 genes is flanked by the PLGRKT gene on one side and the JAK2 on the other side. Note that the cow has completely lost the RLN gene, while it has remained as a truncated pseudogene in sheep, goat, and antelope. *Source:* [106].

Moreover, these authors suggested that relaxin activity in its relationship to sperm motility in domestic species may serve as a possible index for predicting the fertilizing ability of sires. Subsequent studies have demonstrated that relaxin improves sperm motility in a number of species, including the human male [110], boar [111], and bull [112]. Furthermore, others have demonstrated that use of relaxin treatment of seminal plasma induces sperm capacitation and acrosome reaction in boar [113] and domestic bull [114] spermatozoa. Other studies have shown that relaxin treatment improved the fertilizing capabilities of fresh boar spermatozoa [115] and fresh or frozen–thawed spermatozoa of the buffalo bull [116, 117] and stallion [118]. Thus, it is evident from these studies that relaxin may play an important role in sperm motility and function. While the RLN1 gene may be lost in the bovid, the physiological benefits of relaxin lie in its potential use as a semen supplement when using either fresh or frozen–thawed bull spermatozoa to facilitate sperm motility and enhance fertilizing capacity during artificial insemination.

Seminiferous Tubules, Sertoli Cells, and Germinal Tissue

Within segments of the testicular parenchyma lie the seminiferous tubules, originally formed from the primary sex cords. The seminiferous tubules contain the Sertoli cells, also known as the nurse cells of the developing germinal cells, the spermatogonia, which are elongated cells found within the tubules. The Sertoli cells were first described by the Italian physiologist Enrico Sertoli in the early 1860s. Structurally, these cells are very important in that they form the blood–testis barrier, which separates the interstitial blood compartment of the testes from the adluminal compartment of the seminiferous tubules. In addition to Sertoli cells nurturing the development and maturation of the germinal cells through the various stages of spermatogenesis, these cells act as phagocytes by removing or consuming residual cytoplasm and surplus spermatozoa material. Sertoli cells, which are also very active secretory cells, produce anti-Müllerian hormone (AMH), a glycoprotein secreted during early fetal life by the testis to direct the appropriate development of the male reproductive tract by causing the regression of the Müllerian ducts [119, 120]. Mutations of the AMH gene lead to persistent Müllerian duct syndrome in male human patients [121]; whether the same observation is true in cattle has not been reported. However, recent studies successfully employed the use of AMH profiles as a novel biomarker to evaluate the existence of functional cryptorchid testis in Japanese black calves [122]. A more comprehensive review of Sertoli cell cytology may be found in the review by de Kretser and Kerr [33].

In addition, Sertoli cells secrete two closely related protein complexes of the transforming growth factor cytokine superfamily (activin and inhibin) that have opposite regulatory effects on the synthesis and regulation of pituitary FSH secretion. Activin upregulates while inhibin downregulates FSH synthesis and secretion [123, 124]. Both of these hormones are secreted after puberty and play an important role in mammalian reproduction, which is coordinated by assorted neural, neuroendocrine, endocrine, and paracrine cell–cell communication pathways [125]. Activin is also thought to facilitate androgen synthesis in the testis by enhancing LH, thereby promoting spermatogenesis. In male mammals, inhibin production is thought to be promoted by testicular androgens and may function locally in the testis to help regulate spermatogenesis. Much less is known about inhibin in relation to its mechanism of action, but it is thought that it may compete with activin for the binding of the activin receptor and/or binding to the inhibin-specific receptor [124]. However, Phillips [126] in reviewing the literature notes that inhibin not only acts as a feedback regulator of FSH in the male, but also may be an important paracrine and autocrine regulator of testis function. It has been shown from molecular studies that both forms of inhibin (A and B) are produced by the bovine testis and that the proportion of the mature forms of inhibin A and inhibin B increases as bulls age, but that total inhibin production by Sertoli cells decreases [127]. In a later study, Kaneko et al. [128] reported that inhibin A and inhibin B increased in the testis of bulls during postnatal development, and that immunoreactive inhibin A could be detected in the plasma of these bulls. Others have attempted to use blood concentrations of inhibin and gene expression along with other endocrine and genetic markers as a predictor of fertility in Brahman bulls [129]. Unfortunately, there appears to be little information in the current literature on the role activin plays in the bovine male. Its role as a paracrine regulator in the testis is presumed but has not been conclusively demonstrated in domestic species, although *in vitro* studies in porcine Leydig cells would suggest a role in androgen synthesis [126]. However, it is thought to be an important intrafollicular factor in the regulation of follicle selection in the bovine female [130, 131]. A role in inflammatory processes for activin has been postulated [126].

Additional factors secreted by the Sertoli cell include ABP, estradiol (by conversion of testosterone via the aromatase enzyme complex), glial cell-derived neurotrophic factor (GDNF), transferrin [132], and the more recently identified ERM transcription factor. Testicular transferrin and ABP are two glycoproteins produced and secreted into the lumen of the tubule by Sertoli cells, and which have important biological effects on differentiation and maturity of sperm [133, 134]. ABP is a glycoprotein that binds spe-

cifically to testosterone, DHT, and 17β-estradiol. By binding to ABP, the steroid hormones testosterone and DHT are less lipophilic and become more concentrated within the lumen of the seminiferous tubules, thereby helping to facilitate spermatogenesis. ABP synthesis is regulated by FSH activity on the Sertoli cells, which may be enhanced by insulin and testosterone. ABP can especially combine with testosterone to maintain a high concentration of androgen in the seminiferous tubule, which facilitates the development and maturation of spermatogenic cells. Androgen is transferred to the target cells and via the nuclear transfer mechanism into the nucleus to combine with androgen receptors, which regulate translation of the target genes. The characteristic of ABP combining with androgen shows that ABP participates in sexual differentiation and maturation of germ cells [132].

Sertoli cell gene products include growth factors, metabolic enzymes, transport proteins (e.g. transferrin, ABP), inhibin, proteases, antiproteases, energy metabolites, and structural components [135]. Transferrin is a major secretory product of differentiated Sertoli cells and is postulated to transport iron sequestered by the blood–testis barrier to the developing germ cells. Fe^{3+} in blood is transferred to Sertoli cells, combined with transferrin synthesized by Sertoli cells, and further transferred to spermatogenic cells at the development stage to promote germ cell growth and maturation. It is well known that the synthesis and secretion of transferrin and ABP are regulated by FSH and androgen. The gonadotropin FSH is an important endocrine hormone required for the regulation of Sertoli cell function [136]. FSH has been shown to regulate the expression of most Sertoli cell genes, including the FSH receptor, ABP, transferrin, plasminogen activator, and aromatase [137–140].

GDNF is a small protein normally associated with the promotion of neuron survival [141]. Its role in testicular function is not fully understood, but there is some evidence to suggest that it may be involved in promoting undifferentiating spermatogonia, which ensures stem cell self-renewal during the perinatal period [142]. Moreover, Johnston et al. [143] demonstrated in the adult rat that cyclic changes in GDNF expression by Sertoli cells are responsible for the stage-specific replication and differentiation of stem spermatogonia, the foundational cells of spermatogenesis. Recent reports in the literature have demonstrated that GDNF, rather than induce proliferation of spermatogonia stem cells, enhances self-renewal and increases survival rate of bovine spermatogonia in culture [144]. An interesting observation reported by Harikae et al. [145] is a rare case of a freemartin calf exhibiting the transdifferentiation of ovarian somatic cells into testicular somatic cells including Sertoli cells, Leydig cells, and peritubular myoid cells, and that the Sertoli cells stained positive for GDNF protein. They speculate that these observations suggest that mammalian XX ovaries may have a high potential for sexual plasticity. For a more comprehensive review of the function and regulation of Sertoli cells, the reader is referred to the excellent review by Russell and Griswold [146].

Overview of Spermatogenesis and Maturation of Spermatozoa

The first studies on the process of spermatogenesis and the spermatogonia of the domestic bull were reported in the latter part of the nineteenth century [147], but it was not until 1931 that the chromosome number of spermatogonia and spermatocytes was determined and that the diploid number was 60 [148]. Subsequent studies in the era after the Second World War described the germ cells and their cytoplasmic structures in more detail [147]. It is now well established that spermatogenesis is a complex cellular process whereby spermatozoa, the male haploid germ cell or gamete, are formed from the diploid spermatogonia stem cells through a series of cellular transformations. These complex transformations occur in the seminiferous tubules of the mammalian testes and may proceed over an extended period of time, which is species dependent. Spermatogenesis has been described morphologically in distinct and recognizable cellular "stages" or "phases" that progress through highly organized and precisely timed cycles [35]. During fetal development primordial germ cells migrate to the embryonic testes where they undergo mitotic division to form gonocytes. Just prior to puberty, gonocytes differentiate into the primary pool of A0 spermatogonia, the stem cells from which all subsequent classes of spermatogonia arise. Spermatogenesis proceeds through three distinct stages within the seminiferous tubules of the testis. The first stage is spermatocytogenesis, a proliferative phase where spermatogonia undergo a series of mitotic divisions to form primary spermatocytes. The second stage is meiosis where the primary spermatocytes undergo reduction division of the chromosomal number, from primary spermatocytes ($4n$) to secondary spermatocytes which are diploid ($2n$) to the final division that produces round haploid ($1n$) spermatids. The final stage is differentiation of the haploid spermatids in a process referred to as spermiogenesis, where the rounded spermatids undergo a series of metamorphic changes to form the elongated and flagellated spermatozoa [35, 149].

Spermatocytogenesis

The initial proliferation occurs at the basal membrane compartment of the seminiferous tubules where the germ cells reside. Spermatogonia emerge from the basal germ

cell layer, where spermatogonia progress through the differentiation cycle and undergo a series of mitotic divisions, the number of which may be species dependent, forming A1 to A4, I and B spermatogonia. Spermatogonia move forward from the basal lamina of the seminiferous tubules as a cohort connected to each other by intercellular bridges, thereby allowing interconnection of cytoplasm between cells. These cytoplasmic bridges may facilitate intercellular communication and thus support the synchronized development of the cohort of spermatogonia. The bull and ram undergo four mitotic divisions during spermatocytogenesis, yielding 16 primary spermatocytes from each active spermatogonium [18]. During this process, a pool of stem germ cells (dormant spermatogonia A1 and A2) is maintained that provides for new generations of spermatogonia (stem cell renewal). This allows the continual production of spermatozoa in the adult male, but the precise mechanism of this regeneration process or renewal of germinal stem cells is not completely understood [150]. During this process, there may be a level of cell loss resulting from cellular degeneration. These cells are resorbed by Sertoli cell cellular phagocytosis.

Meiosis

Mitotic division provides a continuous supply of spermatogonia, while meiotic division is a process whereby B spermatogonia undergo reduction division to form haploid spermatocytes [35]. This meiotic process not only halves the number of chromosomes in spermatocytes, but also ensures genetic diversity by DNA replication and crossing-over, random events that produce genetically unique individual spermatozoa. This replication and crossing-over of DNA material occurs during the first meiotic division, while the second meiotic division results in the formation of the haploid spermatocytes. Spermatocytes may be found at all stages of development in seminiferous epithelium due to the prolonged nature of the meiotic period of spermatogenesis, which ranges from 18 to 21 days in the bull (Figure 2.4a). At the completion of this meiotic process in bulls, each B spermatogonium will result in the production of four haploid spermatids, with a total of 64 spermatids emerging from a single active (A3) spermatogonium [152–154].

Spermiogenesis

The final phase is the differentiation or transformation of the round spermatids into elongated, flagellated, and highly condensed mature spermatozoa that are released into the seminiferous tubule lumen [35]. This process is remarkably similar across domestic species and involves a complex series of events whereby the spermatid undergoes metamorphosis into a highly organized motile cellular structure [33]. Differentiation consists of four main phases that occur in the adluminal compartment between adjacent Sertoli cells: the Golgi phase, the cap phase, the acrosomal phase, and the maturation phase. During the Golgi phase, small Golgi vesicles within the spermatid cytoplasm fuse to form a larger complex structure, the acrosomic vesicle or acrosome. The acrosome is a membrane-bound vesicle or lysosome that contains several enzymes including acid hydrolase, acrosin, esterases, hyaluronidase, and zona lysine. As differentiation progresses, the acrosome begins to migrate to form a cap (capping) over the nucleus of the cell. During capping, the acrosomic vesicle flattens and covers approximately one-third of the nucleus [35]. At the same time the nucleus and cytoplasm undergo elongation, and the nucleus begins to occupy the head region of the spermatid. While the head region of the spermatid is undergoing elongation, the midpiece and tail are being formed, thus initiating the maturation phase. Mitochondria migrate in the cytoplasm to form a spiral assembly around the flagellum posterior to the nucleus, which defines the midpiece of the spermatid. The flagellum originates from the distal centriole that gives rise to the axoneme, which is composed of nine pairs of microtubules arranged radially around two central filaments [21]. The axoneme is connected to the base of the nucleus and extends through the midpiece and continues on to form the principal piece or "tail" of the spermatid. As the cytoplasm from the spermatid is shed during the formation of the tail, a cytoplasmic droplet is formed on the neck of the spermatozoon. Once the metamorphosis is complete, the flagellated and elongated spermatid is now referred to as a spermatozoon (Figure 2.4b). These metamorphic changes are all important developmental steps to ensure that the spermatozoa have the ability to be not only motile but also capable of fertilization and delivery of nuclear material when they come in contact with the female gamete (oocyte).

Spermiation

The final stage of development results in the release of the spermatozoa into the lumen of the seminiferous tubules by a process referred to as spermiation [33]. As the final stages of spermiogenesis are completed, the spermatozoa emerge from the adluminal compartment of the seminiferous tubules tail toward the apex of the Sertoli cells and the tubule lumen. During the process of migration from the rete testis through the epididymis and ejaculate, the spermatozoa shed their cytoplasmic droplets, but not all emerge as normal spermatozoa in the ejaculate (Figure 2.5). Before spermatozoa are capable of fertilization they must undergo additional maturation in the epididymis. Capacitation, the final stage of maturation where spermatozoa gain the

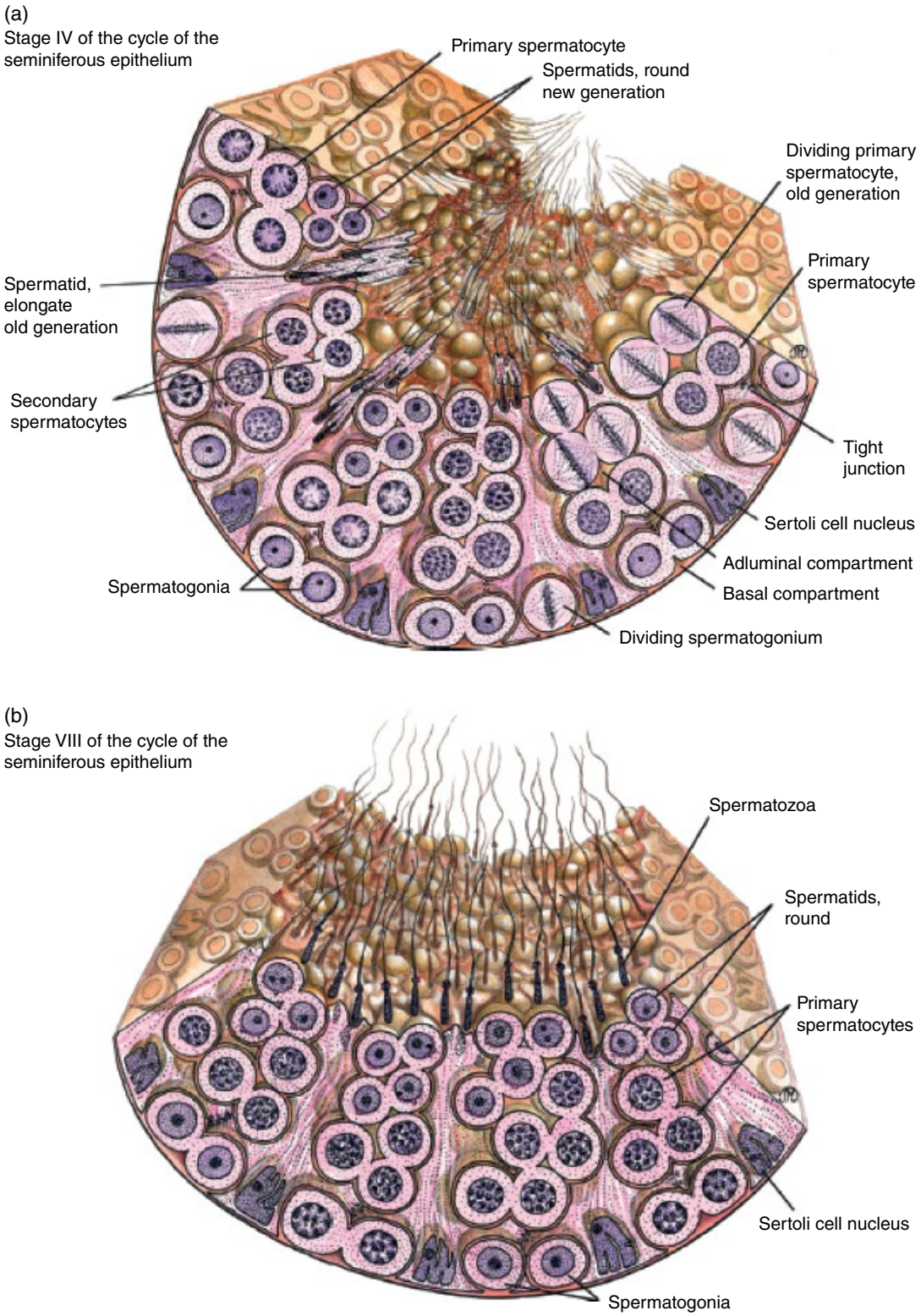


Figure 2.4 Schematic representations of the germinal epithelium of the bull testis during stage IV and stage VIII of the seminiferous epithelium cycle. Stage IV of the cycle (a) is characterized by the very active generation of primary spermatocytes into secondary spermatocytes during meiosis I and progress on with the completion of the meiotic division of meiosis II and the formation of spermatids. Stage VIII of the cycle (b) is characterized by the emerging free spermatozoa that have undergone differentiation from the round spermatids. *Source:* From [151], © 2003, Germinal Dimensions Incorporated.

ability to penetrate the zona pellucida (the outer membrane of the oocyte), occurs in the female reproductive tract. For more specific descriptions of the process of spermatogenesis in the bull the reader is referred to a series of articles by Amann [152], Berndtson and Desjardins [153], and Johnson et al. [154].

Seminiferous Epithelial Cycle and Wave of Spermatogenesis

The cycle of the seminiferous epithelium refers to a cohort of germ cells that progress by clonal expansion within a segment of the seminiferous tubule and change in synchrony with other layers over time through a series of well-characterized cellular associations referred to as stages [35, 149]. Cross-sections along the length of the seminiferous tubules will have different morphological appearances, but within a given section the cellular activity will be similar. Successive sections of the seminiferous tubules will have cohorts of germ cells at sequential stages of development with repetition, thus producing a "wave" effect along the seminiferous tubules [35]. Germ cell development progresses from the basal lamina of the seminiferous tubule, and cells migrate toward the tubule lumen and not in a lat-

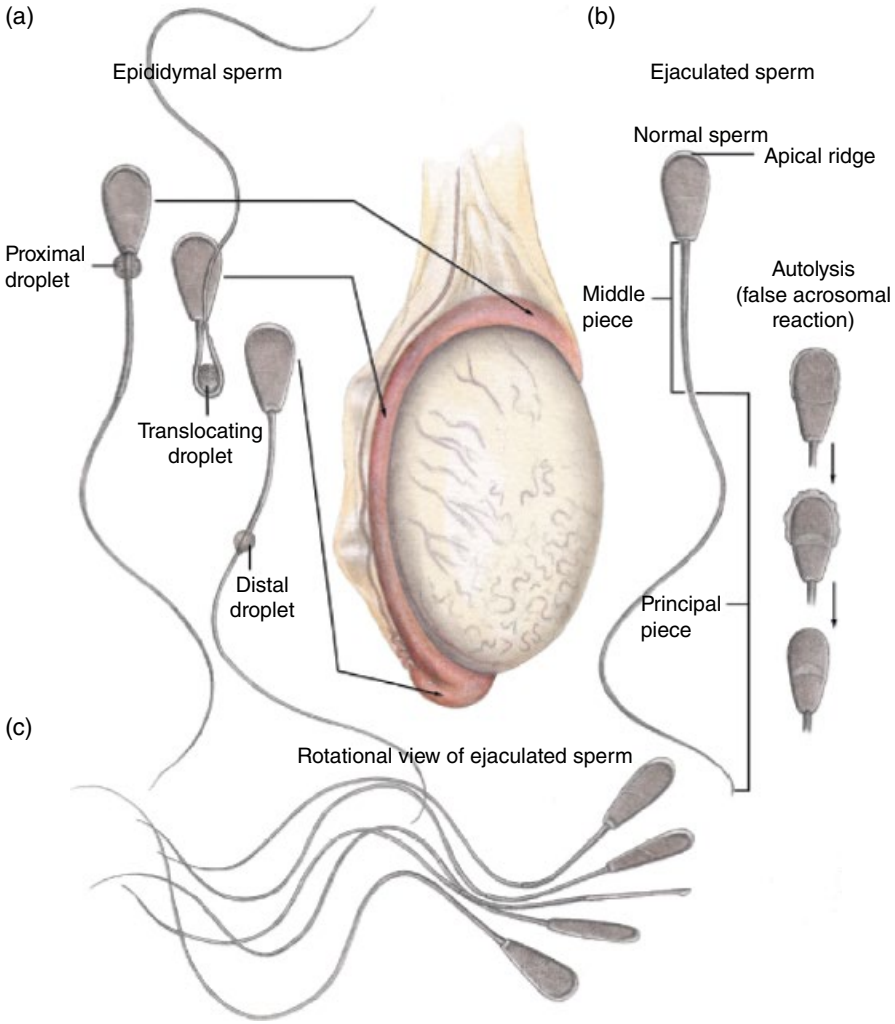


Figure 2.5 Schematic representation of spermatozoa reflecting visible changes as they migrate from the rete testis through the caput (head), corpus (body), and cauda (tail) regions of the epididymis to the ejaculate. Sperm found in the rete testis, efferent ducts, and caput epididymis are identified by the proximal nature of the protoplasmic droplet, while in sperm in the distal caput through to the corpus epididymis the protoplasmic droplet has translocated to the bend of the flagellum; by the time the spermatozoa reach the cauda epididymis the droplet has located at the distal portion of the middle piece of the flagellum (a). Schematic representations of ejaculated normal and autolysis spermatozoa are shown in (b). Normal sperm show an intact acrosome in the form of the apical ridge along the apical edge of the spermatozoan head, while the spermatozoan head images to the right show examples of false or premature acrosomal reactions typically associated with dead or dying spermatozoa. The paddle-shaped nature of the bovine spermatozoon is shown in (c). *Source:* [151], © 2003, Germinal Dimensions Incorporated.

eral direction. This synchronous behavior is thought to be associated with biochemical signaling facilitated by the intercellular bridges, but the process is not fully understood. In the bull, it takes about 4.5 cycles of the seminiferous epithelium to complete spermatogenesis, and each cycle is estimated to take approximately 13.5 days, thus requiring 61 days to complete the process [152, 154]. Spermatogenesis continues throughout the natural lifespan of the postpubertal bull. The domestic bull attains puberty at approximately 10–12 months of age when mature sperm begin to appear in the ejaculate. It is estimated that the daily sperm output of the dairy bull is approximately eight billion and approximately seven billion in the beef bull, with the level of production of spermatozoa increasing from puberty to maximum potential at approximately three years of age. The productivity potential of a bull is determined by the size of the testes, which may be breed dependent, and is thought to be related to being a component of Sertoli cell volume within the seminiferous tubules. Hence, breeding soundness examinations of bulls to be used as sires is dependent not only on sperm quality but also on testicular size and volume [155].

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3

Spermatogenesis

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Introduction

Spermatogenesis is a highly proliferative and regulated developmental process of multiple germ cell divisions to increase their number and subsequently differentiate to spermatozoa in the seminiferous tubules (ST) of testes. Spermatogenesis is essential for species conservation and genetic diversity within the species [1]. For cattle farming, spermatogenesis yields target spermatozoa for genetic improvement in production potential. Traditionally, the bull has been truly called half of the herd on account of his spermatogenesis. A bull produces more calves per year per herd than a cow. The bull contributes to the genetic and production potential of the herd more extensively and perpetually than does the cow. If the replacement heifers are maintained, the bull affects the production potential of the herd for about 25 years [2]. Spread of male germplasm through artificial insemination is the major tool for genetic improvement in cattle production. In the United States, adaptation of artificial insemination led to a 4.5-fold increase in milk production per dairy cow on average from 1940 to 2009 [3].

Cell and Molecular Biology of Spermatogenesis

Spermatogenesis starts at puberty and human beings produce 1000 spermatozoa with each heartbeat. This enormous spermatogenesis is maintained by spermatogonial stem cells (SSCs) at the basement of the ST [4]. Alkaline phosphatase positive epiblast cells of ectoderm are the embryonic origin of SSCs. About a hundred such cells, which had been lineage restricted from day 7.2 post mating, develop to primordial germ cells (PGCs) in the mouse embryo [5]. The PGCs multiply and reach the genital ridge which is prominent by day 11.5 post coitum [6]. About 10 000 unipotent PGCs are found in each mice gonad. In the gonadal ridge, PGCs harbor the seminiferous cords and become mitotically arrested at day 13.5 post coitum, thereafter termed as gonocytes or prespermatogonia or prospermatogonia. Prospermatogonia, morphologically different from PGCs, undergo extensive epigenetic reprogramming for their later functioning as male gametes [7]. Once prospermatogonia reach basement peripartum, they are morphologically and biochemically different from prospermatogonia and are termed as $A_{undifferentiated}$ spermatogonia. $A_{undifferentiated}$ spermatogonia proliferate but remain connected as A_{single}, A_{paired}, and $A_{aligned}$ through intercellular bridges. Intercellular bridges play a role in cell to cell communication and synchronization of these mitoses. $A_{aligned}$ are syncytia of 4, 8, and 16 undifferentiated spermatogonia. Currently, no method distinguishes among different types of $A_{undifferentiated}$ spermatogonia [1]. An undifferentiated spermatogonia irreversibly differentiates to A_1 spermatogonia under the repeated stimulation of retinoic acid. In rodents, chain identity is maintained upon this transition and it has been estimated that 64% of A_{pair}, 94% of $A_{aligned4}$, and 100% of $A_{aligned8\text{-}16}$ transition to the differentiating A_1 state following a retinoic acid pulse [8]. A_1 spermatogonia undergo six synchronized mitoses through A_2, A_3, A_4, intermediate (In), and B spermatogonia to form primary spermatocyte [4]. In the classical Huckins and Oakberg model, SSCs are contained within the A_{single} population [9, 10]. However, one current model of SSC renewal suggests that syncytia of A_{paired} and $A_{aligned}$ spermatogonia can break to yield SSCs [11].

All undifferentiated spermatogonia other than SSCs are called progenitors. A_{single} undergo symmetric divisions forming syncytia of undifferentiating spermatogonia, or there is asymmetric division forming an SSC and a progenitor A_{single}, thus maintaining steady-state spermatogenesis [4]. Total

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

number of A_{single} in adult mouse testis is estimated at 35 000 and each adult mice testis has about 3000 SSCs, which is about 0.01% of total testis cells [12], and 0.03% of the entire testicular germ cell population. Furthermore, 33×10^4 undifferentiated spermatogonia and 280×10^4 differentiating spermatogonia populate each testis in the mouse [8]. SSCs as well as epiblast and PGCs from days 6–16.5 post coitum embryo can regenerate spermatogenesis upon transplantation to appropriate donor testes [13].

SSCs, capable of self-renewal and generating progenitor cells through symmetrical and/or asymmetrical divisions, reside in a specific microenvironment within the ST termed as a germ line stem cell niche [14]. Within this microenvironment of the niche, SSCs are intermingled with other undifferentiated spermatogonia. Sertoli cells in communication with other somatic cells of the testes secrete factors to maintain the microenvironment of the niche. Within the niche, SSCs are located toward areas of the ST closer to interstitial vasculature [15].

Development of preleptotene spermatocyte from type B spermatogonia marks a cell's detachment and migration away from the basement membrane. Preleptotene spermatocytes cross the tight Sertoli cell junctions and reach the adluminal portion of the ST. Primary spermatocytes first divide mitotically and then form short-lived haploid secondary spermatocytes through meiosis I. During prophase of meiosis, preleptotene spermatocytes differentiate into leptotene, zygotene, pachytene, and diplotene, respectively. Haploid spermatids are formed by equational meiosis II of secondary spermatocytes. Round spermatids mature into spermatozoa through various steps of spermiogenesis without any increase in cell number. Spermatozoa are released into the lumen of the ST through the process of spermiation [16]. Figure 3.1 shows the steps in spermatoogenesis.

Spermatogenesis can be divided into spermatocytogenesis, meiosis, and spermiogenesis. During spermatocytogenesis, germ cells divide by several mitoses to increase the yield of spermatogenesis, renew spermatogonial stems cells, produce more undifferentiated spermatogonia, and finally produce primary spermatocytes. During meiosis, recombination of genetic material happens, homologous chromosomes move apart, and chromosome number is reduced by half to yield haploid round spermatids. Spermiogenesis involves differentiation of haploid round spermatids into mature haploid elongated spermatozoa without mitosis or meiosis [16].

Markers to Study Spermatogenesis

Id4, *Gfra1*, *Bcl6b*, *Lhx1*, and *Etv5* are upregulated in SSCs. *Sohlh1*, *Ngn3*, and *Kit* are expressed by progenitor spermatogonia [17]. THY1, ID4, and GFRA1 are markers for SSCs, NGN3 and KIT for progenitors, PLZF/ZBTB16 for all undifferentiated spermatogonia, and VASA for all germ cells [18].

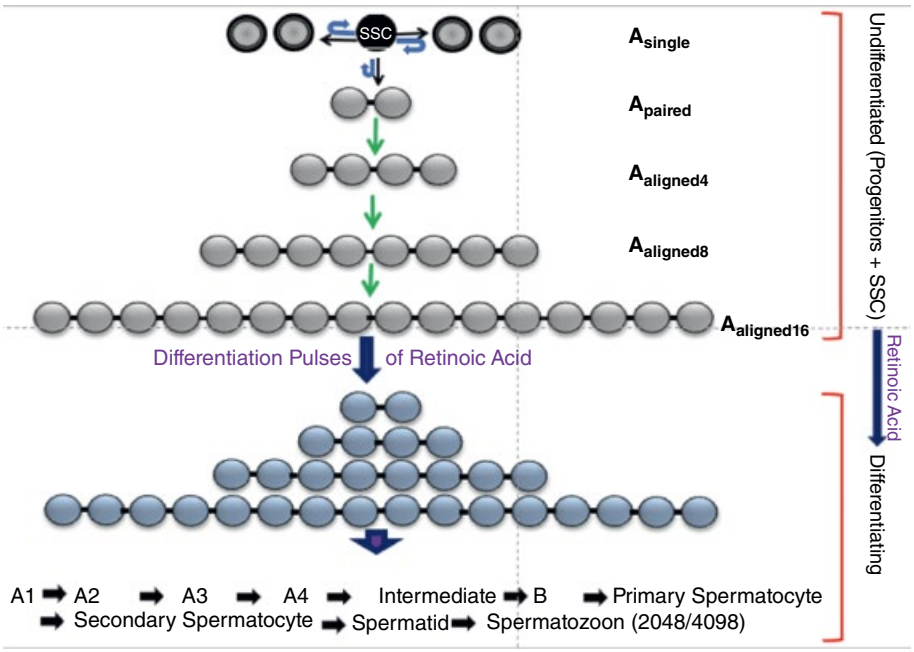


Figure 3.1 Steps in spermatogenesis.

Hormones and Spermatogenesis

Testosterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) are the main hormones required for spermatogenesis. Deficiency of these hormones causes germ cell apoptosis, while the administration of these suppresses apoptosis. Thus, these hormones are the survival factors required by germ cells. Under testosterone deficiency, round spermatids do not transition to elongated spermatids as the spermatids lose their attachment to Sertoli cells. FSH mainly acts indirectly through Sertoli cells. FSH has been linked to early stages in spermatogenesis, mainly spermatocytogenesis and meiosis, while testosterone is linked to later stages of spermatogenesis, mainly in spermatid differentiation and in potentiating the effect of FSH.

The Cycle of the Seminiferous Epithelium

The cycle of the seminiferous epithelium refers to is the histological fact that at a given section of the ST, different types of germ cells appear cyclically at species-specific time intervals [16]. The notion of the cycle of the seminiferous epithelium was established toward the end of the nineteenth century [19]. Several generations of germ cells are present along the ST because spermatogenesis is a continuous process and subsequent spermatogenesis does not pause to wait for completion of the first generation to the end-product spermatozoa and the release of these spermatozoa in the lumen of the ST [20]. $A_{undifferentiated}$ spermatogonia differentiate to $A_{differentiating}$ spermatogonia (A_1 and subsequent spermatogenesis) regularly after a species-specific fixed time interval. This $A_{undifferentiated}$ to A_1 transition does not wait for the preceding process of "$A_{undifferentiated}$ to A_1 transition and finally to spermatozoon" to complete [21]. Along the section of the ST, the development of germ cells is influenced by neighboring cells, resulting in specific germ cell associations following one another in time at a given section of the ST in a perfectly organized pattern, termed the cycle of the seminiferous epithelium. This results from the fact that (i) at specific points of the ST new spermatogonia enter spermatogenesis at constant time intervals, and (ii) once the cells are engaged in spermatogenesis, their rate of differenation to the next cell type is always a constant and each step of spermatogenesis has a constant and fixed time duration. The specific germ cell associations at a given point of the ST during the cycle of the seminiferous epithelium are termed as stages, or less commonly as phases of the cycle. In the bull, the cycle is divided into eight stages and spermiation is taken as the reference point [22, 23]. In bulls, the duration of the seminiferous epithelium cycle is 13.5 days. The total duration of spermatogenesis is 4.5 times the duration of the cycle, that is 61 days. The durations of spermatocytogenesis, meiosis, and spermiogenesis are 21, 23, and 17 days, respectively [16].

The spermatogenic wave is "a sequence of segments showing the complete series of cell associations corresponding to the stages of the cycle of the seminiferous epithelium. One or more modulations must, however, be excluded from the sequence of the segments considered." While the cycle of the seminiferous epithelium refers to the temporal arrangement of the germ cells at a given point of the ST, the spermatogenic wave refers to the spatial arrangement of germ cells along the ST [24]. However, the wave is not in space what the cycle is in time – the wave is not a dynamic process but is a static way to describe the spatial distribution of the associations along the tubule [16]. Spermatogenesis progresses along the ST in wave form, and the wave is formed by the fact that specific germ associations, i.e. stages, start again and again at specific distance. The regulated order of the wave emergence along the ST follows the numerical order of the stages of the cycle of the seminiferous epithelium [25, 26]. Different waves start as we move along the ST, and at a specific section of the ST, cells from different waves are seen together in a cross-section of the ST according to the stage of the cycle of the seminiferous epithelium. The waves move along the ST in an inward spiral fashion, with $A_{1\ undifferentiated}$ at the outer border of the wave and spermatozoa at the inner border of the wave. One spermatogenic wave starts with initiation of spermatogenesis, i.e. from conversion of type $A_{undifferentiated}$ to type A_1 differentiating spermatogonia under the repeated actions of retinoic acid, and ends with formation of spermatozoa [27]. Histologically, stages of the cycle are identified by two methods: (i) by meiotic status of spermatocytes and changes in the shape and position of nuclei of spermatids; or (ii) by differential morphology of the acrosome of spermatids [24].

Spermatogenesis in the Bull

Spermatogenesis in the bull establishes progressively from 16 weeks to 32 weeks of age. Gonocytes are the main germ cells in the ST until one week of age and are sequentially replaced by undifferentiated spermatogonia by 20 weeks. Meiosis starts at about 16 weeks and is completed by 28–32 weeks. Complete spermatogenesis can be observed in cross-sections of the ST by 32 weeks of age [23].

Histologically, spermatogenesis is evaluated qualitatively by the appearance of the ST and quantitatively by differen-

tial cell count in the ST. Daily sperm production (DSP, millions) per gram of decapsulated testes is an appropriate measure of the efficiency of spermatogenesis and is used for species comparison [28]. DSP per gram of decapsulated testes is 4–6 for the human man, 12 for the bull, 16–19 for the stallion, 21 for the ram, 23 for the rhesus monkey and for the boar, 20–24 for the rat, 24 for the hamster, and 25 for the rabbit [28–30]. DSP is affected by longer duration of spermatogenesis, longer cycle of the seminiferous epithelium, the density of germ cells in the ST, and germ cell degeneration during spermatogenesis [30]. The reason for lower efficiency of spermatogenesis in the bull is not understood [16]. In the bull, germ cell degeneration during transition from A_1 to A_4 and at intermediate spermatogonia stage accounts for 30% losses in DSP. Moreover, degeneration at B_1 and during transition from B_1 to B_2 causes another 30% loss. However, there is no loss during meiosis and spermiogenesis in the bull. In the absence of this degeneration, the bull would have a DSP of around 30×10^6/g of testes [16, 25].

When a bull can produce an ejaculate with 50 million spermatozoa with at least 10% motility, he is pubertal [31]. Age at puberty varies from 38 to 48 weeks of age, with an average age of 42 weeks [32]. After maturity, a bull can give two useable ejaculates daily; commercially, bull ejaculates are collected three times a week for a balance between management and number of artificial insemination doses. Both the quality and quantity of spermatogenesis decrease in old age; however, the aging-related extinction of spermatogenesis in the bull has not been reported. Aging-related decrease in sperm production has not been studied in the bull as breeding bulls are not maintained for senescence. One of the reasons for age-related decline in spermatogenesis is decreased Sertoli cell number in old age [16].

Sertoli Cells and Spermatogenesis

Sertoli cells (SC) play an indispensable role in the regulation of spermatogenesis, establishing the rate of spermatogenesis, and in development and movement of germ cells [33, 34]. SC are one of the most complex and dynamic cells in biology [35].

The cell was first described by and hence named after Enrico Sertoli [36]. SC are large irregular shaped columnar cells extending from the base of the ST to the apex of the ST and occupy 17–19% volume of the tubule [34]. The large surface area of SC allows for interaction with an enormous number of germ cells as the SC to germ cell ratio in adult rats is about 1 : 50 [37]. SC are the most important somatic cells of testes and possess high plasticity synchronized with cyclic evolution of germ cells. The cells change their structure during their development and according to the cycle of the seminiferous epithelium. At any life point, SC of type A and type B are seen in the ST. Type A SC have cytoplasmic crypts for attachment of mature spermatids ready for release into the ST lumen. In type B SC such cytoplasmic crypts are less prominent or absent [38]. The SC nucleus is multilobed and the cytoplasm is rich in endoplasmic reticulum, glycoproteins, and cytoplasmic droplets; the cytoskeleton varies with the cycle of the seminiferous epithelium [39].

The Role of Sertoli Cells in Spermatogenesis

Structural Support

SC in association with peritubular myoid cells secrete basement membrane of the ST [40]. The cytoskeleton of SC participates in organizing and shaping the ST [41]. Major components of SC cytoskeleton are actin, intermediate filaments, and microtubules, and each has a unique distribution pattern according to different stages of the cycle of the seminiferous epithelium [42]. The functions of SC cytoskeleton include (i) maintaining SC shape; (ii) positioning and transporting organelles within the cell; (iii) forming and stabilizing SC membrane at sites of cell–cell and cell–extracellular matrix contact; (iv) positioning, anchoring, and aiding in the movement of developing germ cells; and (v) involvement in the release of mature spermatids from the ST during spermiation [34].

Blood–Testes Barrier

At puberty, neighboring SC develop tight junctions, forming an impermeable blood–testes barrier [40]. The barrier is a modified occluding junction located in the basal third of the seminiferous epithelium [43]. The blood–testes barrier divides the seminiferous epithelium into basal and adluminal compartments [44]. The basal part houses spermatogonia, preleptotene, and leptotene spermatocytes, while further advanced meiotic spermatocytes and spermatids reside in the adluminal part [34].

The blood–testes barrier shields germ cells in the adluminal part of the ST from direct contact with blood, protecting the cells from toxic, mutagenic, and autoimmune reactions. The barrier maintains specific concentrations of androgen-binding protein, inhibin, activin, and enzyme inhibitors within the adluminal compartment of the ST.

It controls transport of molecules and wastes to and from the adluminal compartment [45, 46]. The blood–testis barrier also functions as an immunological barrier to protect novel proteins at spermatocytes and spermatids from autoimmune reaction and inhibits immunoglobulins and lymphocytes from entering the adluminal part [35]. SC and germ cells produce interferons, interferon-induced proteins, interleukins, and cytokines, which maintain an antiviral defense system [47].

Germ Cell Translocation

As germ cells lack the necessary architecture for migration, SC are responsible for germ cell migration across the seminiferous epithelium. SC assist in the translocation of preleptotene spermatocyte from the basal to adluminal compartment of the ST across the blood–testis barrier [34]. Specialized adhesion junctions, ectoplasmic specializations, of SC attach to the spermatid head and translocate spermatids up and down across the seminiferous epithelium. Ectoplasmic specializations consist of regions of SC plasma membrane adherent to the spermatid head, a submembrane layer of tightly packed SC actin filaments, and an attached cistern of SC endoplasmic reticulum. Motor proteins of SC endoplasmic reticulum translocate ectoplasmic specializations and hence spermatids are also translocated [48].

Spermiation

During spermiation, mature spermatids are released from SC into ST lumen prior to their subsequent passage to the epididymis. For spermiation, mature spermatids are oriented toward the lumen of the ST and subsequently released into the lumen by spermiation machinery over several days. Spermiation involves remodeling of the spermatid head and cytoplasm, removal of specialized adhesion structures between SC and mature spermatids, and the final expulsion of mature spermatid heads from the SC crypts [49].

Phagocytosis

After spermiation, SC phagocytose degenerated residual bodies of released spermatids. Moreover, SC phagocytose the germ cell degenerated during spermatogenesis [50]. Moreover, SC are capable of pinocytosis in the adluminal compartment and receptor-mediated endocytosis in the basal compartment of the ST [51].

Secretions

SC secrete different types of proteases, protease inhibitors, hormones, energy substrates, growth factors, paracrine factors, and extracellular matrix components. Proteases and protease inhibitors from SC participate in ST maintenance, repair, growth, remodeling, and restructuring. Protease inhibitors are required for germ cell translocation across the blood–testis barrier and for spermiation [52]. Moreover, protease inhibitors are involved in assembly and disassembly of cellular junctions in the ST [53].

SC secrete adluminal fluid and maintain a specific environment for differentiation of germ cells toward lumen of the ST [33]. FSH-regulated functions of SC include transfer of testosterone and glucose and germ cell nurturing by provision of lactate and pyruvate. Under the influence of FSH and androgens, SC secrete proteins necessary for germ cells and interstitial cells of the testes and secrete androgen binding proteins to ensure bioavailability of androgens [54, 55]. Inhibin and activins from SC act on the hypothalamic–pituitary–gonadal axis and also on Leydig cells [56]. The iron carrier protein, transferrin, is produced by SC and its concentration in seminal plasma is correlated with fertility and spermatogenic capacity in the bull [57]. SC co-cultured with germ cells stimulate DNA and RNA synthesis in the germ cells [58]. Seminiferous growth factor is involved in spermatogonial proliferation, nerve growth factor is required for DNA synthesis in preleptotene spermatocytes, and insulin-like growth factor-1 helps in germ cell differentiation [59].

The germ cells residing in the adluminal compartment cannot receive nutrients directly from blood due to the blood–testis barrier. The spermatocytes and spermatids in the adluminal compartment are nursed by amino acids, carbohydrates, lipids, vitamins, and metal ions from SC [34].

The Junctional Role of Sertoli Cells

SC make and break junctions among themselves and with different types of germ cells. Major junctions formed by SC are tight junctions, anchoring junctions, ectoplasmic specialization, tubulobulbar complexes, and communication junctions. Tight junctions are present between adjacent SC at the level of the blood–testis barrier and function as a semipermeable barrier – the barrier function. Tight junctions divide SC into basal and adluminal, preventing mixing of molecules in the two compartments – the fence function. Anchoring junctions include adherens junctions (also known as zonula adherens), focal contacts or adhesions, desmosomes, and hemidesmosomes. All these anchoring junctions are biochemically and structurally different from one another and mainly function to connect adjacent cells to one another or to extracellular matrix through cytoskeleton, thus maintaining tissue integrity. Anchoring junctions also function in signal transduction to regulate

cell proliferation, differentiation, and translocation. Ectoplasmic specialization is a modified form of adherent junctions and mainly functions in spermiation and maintenance of other junctions present between adjacent SC and, SC and germ cells. Tubulobulbar complexes, also a modified form of zonula adherens, are present between adjacent SC at the level of tight junctions and between SC and mature spermatids ready for spermiation. Focal contacts are actin-based junctions between testicular cells and extracellular matrix and play an adhesive function at ectoplasmic specialization. Desmosomes are intermediate filament-based junctions found between SC and SC, and SC and germ cells. Hemidesmosomes are intermediate filament-based junctions found only between SC and basal lamina; these have adhesive function. Communication junctions are either chemical synapses or gap junctions. Gap junctions, intercellular channels formed by connexons, transduce signals between SC and germ cells and thus participate in germ cell translocation. Gap junctions are present between SC and SC, SC and germ cells, and between two Leydig cells [34].

Sertoli Cells and Spermatogenic Capacity

In the bull, DSP is correlated with total SC number (R = +0.83) and SC per gram (R = +0.47) but is not correlated with the number of germ cells supported per SC. Testicular parenchyma weight is also correlated with total SC number (R = +0.61). Total SC number is the foremost determinant of the variation in DSP among bulls (R^2 = 68.2%) [60, 61]. However, total SC number and the number of germ cells per SC are not correlated with the quality of either fresh or frozen bull semen [62]. Moreover, SC number in the horse (R^2 = 68) and human (R^2 = 39) is also correlated with DSP. The association between SC and DSP goes back to the association between SC and the number of A_1 spermatogonia (R^2 = 55 for the horse) [33].

In the bull, the majority of SC proliferation happens during fetal life in utero. SC number increases by five times from birth until puberty and no increase in SC number occurs after puberty. Once mitosis in SC stops, the cells differentiate to mature SC and continue their adult function for the reproductive life of the bull [63]. However, the stallion being a seasonal breeder has no permanent SC number after puberty and SC number and volume of SC nuclei per testes increase during the breeding season for a 4- to 20-year-old stallion. The size of horse SC does not differentiate with respect to breeding season [33]. A concurrent increase in the number of A spermatogonia accompanies increased SC number during the breeding season [64].

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4

Thermoregulation of the Testes

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Introduction

Since one bull may be responsible for breeding twenty (by natural service in one breeding season) to thousands (artificial insemination) of females, bull fertility is critically important. Although sterile bulls (total inability to reproduce) are uncommon, there can be a wide range in bull fertility, particularly in the absence of selection for fertility [1]. It is well established that a bull's testes must be 2–6 °C cooler than core body temperature for fertile sperm to be produced; consequently, increased testicular temperature, regardless of cause, reduces semen quality [2]. Although the underlying cause of infertility in bulls is frequently unknown, we speculate that it is often increased testicular temperature.

Anatomy and Physiology

Regulation of testicular temperature is dependent on several features. Scrotal skin is typically thin, with minimal hair and an extensive subcutaneous vasculature, facilitating heat loss by radiation [3]. The scrotal neck is the warmest part of the scrotum; a long distinct scrotal neck (and pendulous scrotum) reduces testicular temperature by increasing the area for radiation and enabling the testes to move away from the body. The tunica dartos, a thin sheet of smooth muscle under the scrotal skin, is controlled by sympathetic nerves and contracts and relaxes in cold and warm environments, respectively [4]. The cremaster muscle also contracts to draw the testes closer to the body under cold ambient conditions [4]. Dorsal to the testis is the testicular vascular cone [5], consisting of the highly coiled testicular artery surrounded by the pampiniform plexus, a complex venous network. The testicular vascular cone functions as a classic countercurrent heat exchanger, transferring heat from the artery to the vein, contributing to testicular cooling. Characteristics of scrotal surface temperatures and the testicular vascular cone in bulls aged 0.5–3 years were reported [6]. As bulls age, testicular vascular cone diameter increased; furthermore, increases in testicular vascular cone diameter and a shorter distance between arterial and venous blood in this structure were associated with increased percentage of normal sperm and fewer sperm with defects [7]. In a comparative study of semen quality and scrotal/testicular thermoregulation in *Bos indicus*, *Bos taurus*, and *B. indicus*/*B. taurus* crossbred bulls, there were significant differences among these genotypes in the vascular arrangement, characteristics of the testicular artery (e.g. wall thickness), and thickness of the tunica albuginea; overall, *B. indicus* bulls had the best thermoregulatory capacity whereas *B. taurus* bulls had the worst, with crossbred bulls intermediate [8].

Sweating and whole-body responses also contribute to testicular cooling. In bulls, sweat gland density is highest in the scrotal skin [9]. In rams, apocrine sweat glands in the scrotum discharge simultaneously (up to 10 times per hour) when scrotal surface temperature is ~35.5 °C [10]. In these animals, respiration rate increases in association with scrotal surface temperature, reaching 200 breaths per minute when scrotal surface temperature is 38–40 °C [11].

Surface and Internal Temperatures

In beef bulls, average temperatures at the top, middle, and bottom were 30.4, 29.8, and 28.8 °C (scrotal surface); 33.3, 33.0, and 32.9 °C (scrotal subcutaneous); and 34.3, 34.3, and 34.5 °C (intratesticular), respectively [12]. Therefore, top-to-bottom differences (gradients) in temperature were 1.6, 0.4, and –0.2 °C for scrotal surface, scrotal subcutaneous, and

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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intratesticular temperatures, respectively. Moving dorsal to ventral, the scrotum gets cooler, whereas the testis (independent of the scrotum) gets warmer. These temperature gradients are consistent with their vasculature, as the scrotum is vascularized from top to bottom, whereas the testis is essentially vascularized from bottom to top. In that regard, the testicular artery exits the bottom of the testicular vascular cone, courses the length of the testis (under the corpus epididymis), and at the bottom of the testis ramifies into multiple branches that spread dorsally and laterally across the surface of the testis before entering the testicular parenchyma [13]. Blood within the testicular artery was a similar temperature at the top of the testis compared to the bottom of the testis, but was significantly cooler at the point of entry into the testicular parenchyma (intra-arterial temperatures at these locations were 34.3, 33.4, and 31.7 °C, respectively [14]). Therefore both the scrotum and the testis are warmest at the origin of their blood supply (top of scrotum and bottom of testis), but they both get cooler distal to that point (i.e. bottom of scrotum, top of testis). Remarkably, these opposing temperature gradients collectively result in a nearly uniform intratesticular temperature in situ [15].

In beef bulls, internal temperatures of the caput, corpus, and cauda epididymis averaged 35.6, 34.6, and 33.1 °C (gradient, 2.5 °C), respectively [12]. That the caput was warmer than the testicular parenchyma at the top of the testis was attributed to the proximity of the caput to the testicular vascular cone. Furthermore, it was noteworthy that the cauda, critical in sperm storage and maturation, was cooler than testicular parenchyma.

Bulls fed moderate-energy diets after weaning have better semen quality than those fed high-energy diets. In one study, beef bulls fed a moderate- versus high-energy diet for 168 days after weaning had a larger scrotal surface temperature gradient (3.9 vs 3.4 °C, $P < 0.02$), more morphologically normal sperm (68.8 vs 62.5%, $P < 0.01$), and a higher proportion of progressively motile sperm (53.4 vs 44.5%, $P < 0.006$) [16]. Perhaps increased dietary energy reduced heat loss, thereby increasing temperatures of the testes and scrotum.

Sources of Testicular Heat

Testicular blood flow and O_2 uptake were measured in eight Angus bulls to determine the relative importance of blood flow versus metabolism as sources of testicular heat [17]. Blood flow in the testicular artery averaged 12.4 ml/min. Arterial blood was warmer (39.2 vs 36.9 °C, $P < 0.001$) and had more hemoglobin saturated with O_2 than blood in the testicular vein (95.3 vs 42.0%, $P < 0.001$). Based on blood flow and hemoglobin saturation, the O_2 used by one testis (1.2 ml/min) was calculated to produce 5.8 cal of heat per minute, compared to 28.3 cal/min attributed to blood flow. Therefore blood flow is the major source of testicular heat.

Pathogenesis of Heat-Induced Changes in Sperm Morphology

There is a long-standing paradigm that testes operate in near hypoxia, blood flow does not significantly increase as testes are warmed, and hypoxia disrupts spermatogenesis after increased testicular temperature [18]. However, this had apparently never been rigorously examined until we conducted two studies to test the following hypotheses: (i) hypoxia disrupts sperm quality and production; and (ii) hyperoxia prevents hyperthermia-induced reductions in sperm quality and production.

In one study [19], we exposed 18 rams (nine had an insulated scrotum) to air containing 14, 21, or 85% O_2 for approximately 30 hours. In that study, scrotal insulation (to increase testicular temperature) substantially reduced sperm motility (from 58 to 30%) and proportion of morphologically normal sperm (from 87 to 30%), but effects due to O_2 were minimal. In a second study [20], 96 male CD-1 mice were maintained at 20 vs 36 °C, exposed to 13, 21, or 95% O_2 twice for 12-hour intervals (separated by 12 hours at room temperature and 21% O_2), and euthanized 14 or 20 days after exposure. Interestingly, sperm morphology and specific stages of sperm cell development were altered in mice exposed to 36 °C, including increases in percentage of sperm with defective heads ($P < 0.0001$) or tails ($P < 0.001$) and percentage of altered elongated spermatids ($P < 0.001$). Regarding effects due to O_2 variations, seminiferous tubule diameter and epididymal sperm reserves were reduced in the 13% O_2 group, but sperm quality and production were not consistently disrupted by hypoxia. In addition, no hyperthermia-induced disruptions were prevented by hyperoxia, indicating a major role of increased temperature, but not hypoxia. There were primarily main effects of temperature; mice exposed to 36 °C had smaller testes, fewer morphologically normal sperm, and histologically increased altered spermatids and altered germ cells compared to mice exposed to 20 °C. In both studies, our hypotheses were not supported; sperm quality and production were not consistently disrupted by hypoxia and hyperoxia did not protect against hyperthermia in mice.

We recently conducted two studies in rams under general anesthesia to determine effects of hypoxia and of testicular hyperthermia on testicular blood flow, and O_2 delivery and uptake. In the first study [21], eight rams were exposed to successive decreases in O_2 concentration in inspired air (100, 21, and 13%; 45 minutes at each concentration). As O_2

concentration decreased (100 to 13%), testicular blood flow increased (9.6 vs 12.9 ml/min/100 g of testis, $P < 0.05$). Increased testicular blood flow maintained O_2 delivery and increased testicular temperature by ~1 °C. In the second experiment [22], testicular temperatures of nine crossbred rams were sequentially maintained at 33–35, 37, and 40 °C (45 minutes at each temperature). As testicular temperature increased from 33–35 to 40 °C, there were increases in mean testicular blood flow (9.8 vs 12.2 ml/min/100 g of testes, $P < 0.05$), O_2 extraction (31.2 vs 47.3%, $P < 0.0001$), and O_2 use (0.35 vs 0.64 ml/min/100 g of testes, $P < 0.0001$). In both experiments, there was no evidence of anaerobic metabolism, based on no significant difference in lactate, pH, HCO_3–, and base excess.

Following our studies in rams, we conducted another study to determine the effects of short-term testicular hyperthermia on testicular blood flow, O_2 delivery and uptake, and evidence of testicular hypoxia in pubertal Angus (*B. taurus*) and Nelore (*B. indicus*) bulls (nine per breed) under isoflurane anesthesia [23]. As testes were warmed from 34 to 40 °C, there were increases ($P < 0.0001$, but no breed effects) in testicular blood flow (mean ± SEM, 9.59 ± 0.10 vs 17.67 ± 0.29 ml/min/100 g, respectively), O_2 delivery (1.79 ± 0.06 vs 3.44 ± 0.11 ml O_2/min/100 g), and O_2 consumption (0.69 ± 0.07 vs 1.25 ± 0.54 ml O_2/min/100 g), but no indications of testicular hypoxia (Figure 4.1). Our hypothesis that Angus bulls have a greater relative increase in testicular blood flow than Nelore in response to increased testicular temperature was not supported, as there was no significant breed difference.

Our studies in conscious rams and mice, as well as in anesthetized rams and bulls, challenged the classical paradigm regarding scrotal/testicular thermoregulation, as acute testicular hyperthermia caused increases in blood flow and in delivery and uptake of O_2, with no indications of hypoxia. In contrast to the long-standing paradigm, our data were evidence that effects of increased testicular temperature were due to testicular temperature per se and not secondary hypoxia.

Evaluation of Scrotal Surface Temperature with Infrared Thermography

Infrared thermograms of the scrotum of bulls with apparently normal scrotal thermoregulation were symmetrical left to right, with temperature at the top 4–6 °C warmer than at the bottom [24, 25]. More random temperature patterns, including a lack of horizontal symmetry and areas of increased scrotal surface temperature, were interpreted as abnormal thermoregulation of the testes or epididymides. Nearly all bulls with an abnormal thermogram had reduced semen quality [24, 25]; conversely, not every bull with poor quality semen had an abnormal thermogram. Consequently, infrared thermography is a useful tool for breeding soundness evaluation of bulls, although it does not replace collection and evaluation of semen. In one study, 30 yearling beef bulls, all deemed breeding sound on a standard breeding soundness examination, were individually exposed to approximately 18 heifers for 45 days [26]. Pregnancy rates 80 days after the end of the breeding season were similar (83 vs 85%) for bulls with a normal or questionable scrotal surface temperature pattern, respectively, but were higher ($P < 0.01$) than pregnancy rates for bulls with an abnormal scrotal surface temperature pattern (68%).

Effects of Increased Testicular Temperature

Increased Ambient Temperature

Effects of increased ambient temperature on semen quality have been widely reported. In one study, ambient temperatures of 40 °C at a relative humidity of 35–45% for 12 hours reduced semen quality [27]. Furthermore, *B. taurus* bulls are more susceptible than *B. indicus* bulls to high ambient temperatures [27]. In that regard, decreases in semen quality were less severe, occurred later, and recovered more rapidly in crossbred (*B. indicus* × *B. taurus*) bulls than in purebred *B. taurus* bulls exposed to high ambient temperatures [28].

Scrotal Insulation as a Model of Increased Testicular Temperature

Scrotal insulation is frequently used to increase testicular temperature. In one study [29], scrota of *B. indicus* × *B. taurus* bulls were insulated for 48 hours. The nature and time (day 0, start of insulation) of the morphologically abnormal sperm that resulted were as follows: decapitated, days 6–14; abnormal acrosomes, days 12–23; abnormal tails, days 12–23; and protoplasmic droplets, days 17–23. Therefore scrotal heating affected sperm in the caput epididymis as well as spermatids. Although daily sperm production was not affected, epididymal sperm reserves were reduced by nearly 50% (9.2 vs 17.4 billion), particularly in the caput (3.8 vs 6.6 billion) and cauda (3.7 vs 9.5 billion), perhaps due to selective resorption of abnormal sperm in the rete testis and excurrent ducts. In another study [30, 31], scrota of six Holstein bulls were insulated for 48 hours (day 0, initiation of insulation). The number of sperm collected was not significantly different, but the proportion of progressively motile sperm decreased from 69% (prior to insulation) to 42% on day 15. The proportion of normal sperm

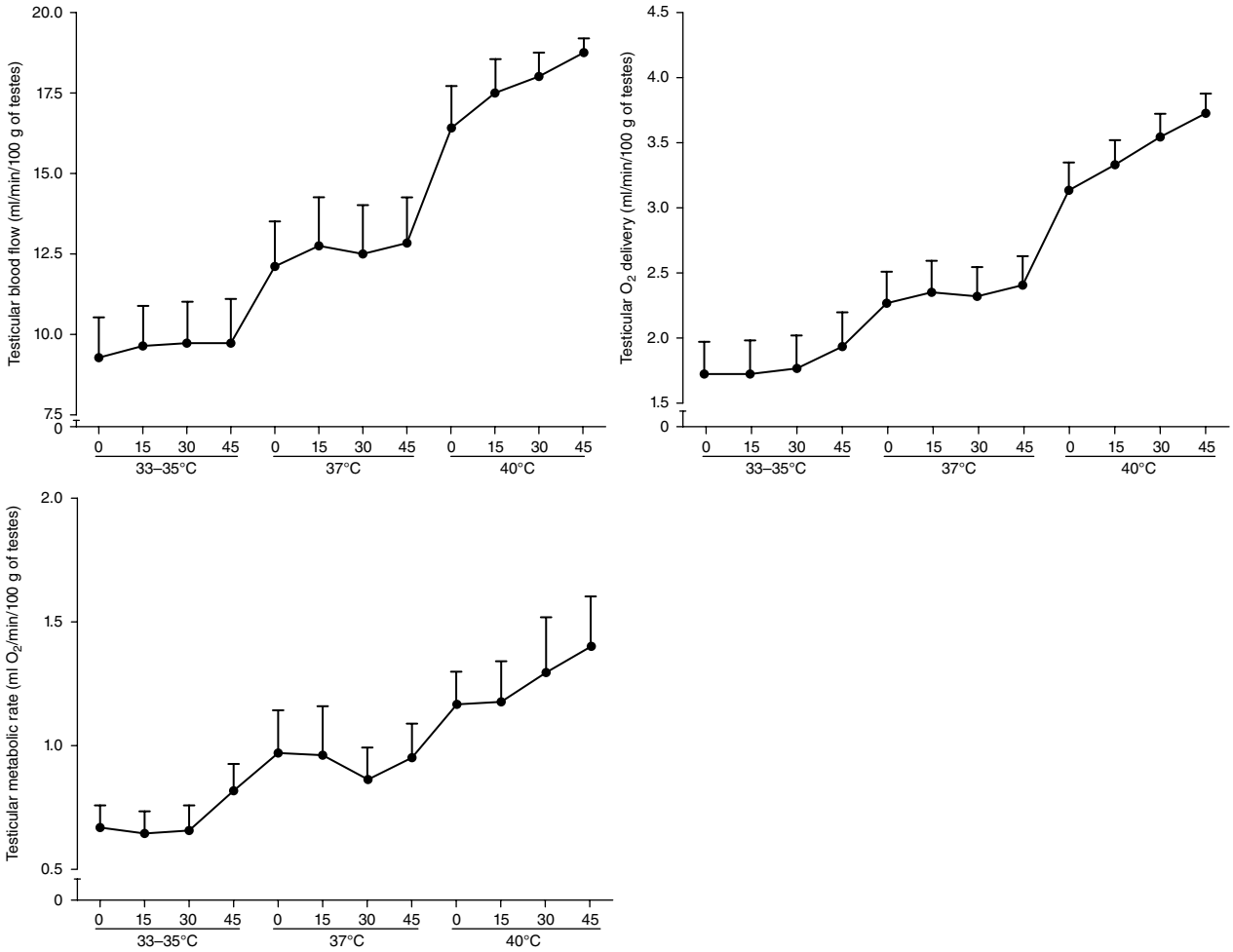


Figure 4.1 Mean (and SEM) blood flow, O_2 delivery, and metabolic rate in testes of 18 bulls (Nelore and Angus) sequentially exposed to three plateaus of testicular temperature (33-35, 37, and 40 °C). Assessment of blood flow and sample collection were done four times at 15-minute intervals and then the testes warmed to reach the next temperature plateau. For each end point, there was a difference ($P < 0.001$) between all temperature plateaus. Testicular temperature increased testicular metabolism; however, testicular blood flow nearly doubled, providing ample O_2 to meet metabolic demands, with no evidence of hypoxia.

was not significantly different from day −6 to day 9 (80%), decreased abruptly on day 12 (53%), and reached a nadir on day 18 (14%). Although there was considerable variation among bulls in both type and proportion of abnormal sperm, specific abnormalities appeared in a consistent chronological sequence: tailless, days 12–15; diadem, day 18; pyriform and nuclear vacuoles, day 21; knobbed acrosome, day 27; and Dag defect, day 30. When sperm were collected 3–9 days after insulation and examined immediately, their motility and morphology were similar to pre-insulation values [30]. Compared to semen collected prior to insulation, following freezing, thawing, and incubation at 37 °C for three hours [30], there were significant reductions in the proportion of progressively motile sperm (46 vs 31%, respectively) and the proportion of sperm with intact acrosomes (73 vs 63%). Freezing plus post-thaw incubation manifested changes that had occurred in sperm that were in the epididymis at the time of scrotal insulation.

In another study [32], scrotal insulation (four days) and dexamethasone treatment (20 mg/day for seven days) were used as models of testicular heating and stress, respectively. Some bulls seemed predisposed to produce sperm with a specific abnormality. Pyriform heads, nuclear vacuoles, microcephalic sperm, and abnormal DNA condensation were more common in insulated than dexamethasone-treated bulls. Conversely, dexamethasone treatment resulted in an earlier and more severe effect on epididymal sperm, an earlier and greater increase in distal midpiece reflexes, and an earlier increase in proximal and distal droplets. Overall, types of defective sperm and the time of their detection were similar between treatments.

Insulation of the Scrotal Neck

The scrotal neck of five bulls was insulated for seven days (days 1–8) as a model of bulls with excessive body condition (which typically have considerable fat in the scrotal neck). Sperm within the epididymis or at the acrosome phase during insulation appeared to be most affected [33]. Insulated bulls had twice as many sperm with midpiece defects and four times as many with droplets on day 5, fewer normal sperm and three times as many with midpiece defects and droplets on day 8, fewer normal sperm on days 15 and 18, and more sperm with head defects on days 18 and 21. Semen quality in insulated bulls had nearly returned to pre-insulation values by day 35. In a second experiment [33], scrotal subcutaneous temperature increased 2.0, 1.5, and 0.5 °C at the top, middle, and bottom of the testis, respectively, and intratesticular temperature was 0.9 °C higher at the corresponding three locations 48 hours after scrotal neck insulation compared to before insulation. Clearly, the scrotal neck is an important site of heat loss.

Increased Epididymal Temperature

In most mammals, the cauda epididymis is cooler than the testes [34], facilitating its sperm storage function. Increasing cauda temperature disrupts absorptive and secretory functions, changes the composition (ions and proteins) of the cauda fluid, and increases (approximately threefold) the rate of sperm passage through the cauda [34]. Consequently, the number of sperm in the first ejaculate declines, with an even more dramatic decline in sperm number in successive ejaculates. In addition, increased temperature seems to hasten sperm maturation [34].

Effects of Increased Temperature on Testicular Cells

Although heating seems to affect Sertoli and Leydig cell function, germ cells are the most sensitive [35]. All stages of spermatogenesis are susceptible, with degree of damage related to the extent and duration of the increased temperature [35]. Spermatocytes in meiotic prophase are killed by heat, whereas sperm that are more mature usually have metabolic and structural abnormalities [36]. Heating testes usually decreases the proportion of progressively motile and live sperm, and increases the incidence of morphologically abnormal sperm, especially those with defective heads [37]. Increased testicular temperature caused a lack of chromatin protamination and subtle changes in head shape of bull sperm [38]. Despite considerable variation among bulls in the nature and proportion of defective sperm, order of appearance of specific defects is relatively consistent [31, 32]. Unless spermatogonia are affected, the interval from cessation of heating to restoration of normal sperm in the ejaculate corresponds to the interval from the beginning of differentiation to ejaculation [37]. Following scrotal insulation in bulls, blastocyst rate (*in vitro* system) was more sensitive than cleavage rate [39]. Even though sperm morphology has returned to normal, their utilization may result in decreased fertilization rates and an increased incidence of embryonic death [40].

Summary

When scrotal/testicular temperature is increased (regardless of the cause), sperm morphology is generally unaffected initially (for an interval corresponding to epididymal transit time) but subsequently declines. In some studies [29, 33], sperm in the epididymis at the time of scrotal heating were morphologically abnormal when collected soon after heating. In another study [30], changes in these sperm were manifest only after they were frozen, thawed, and incubated. Sperm morphology usually returns to pre-treatment values within approximately six weeks after the thermal insult. However, a prolonged and/or severe increase in testicular temperature will increase the interval for recovery. In general, the decrease in semen quality following increased testicular temperature is related to severity and duration of the thermal insult.

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5

Endocrine Control of Testicular Development and Initiation of Spermatogenesis in Bulls

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Introduction

The process of testicular development that leads to initiation of spermatogenesis in bulls involves complex maturation mechanisms of the hypothalamus–pituitary–testes axis. Sexual development can be divided into three periods according to changes in gonadotropin and testosterone concentrations, namely the infantile, prepubertal, and pubertal periods. These changes are accompanied by changes in testicular cell proliferation and differentiation (Figure 5.1).

Infantile Period

The infantile period is characterized by low gonadotropin and testosterone secretion and relatively few changes in testicular cellular composition. This period extends from birth until approximately two months of age in *Bos taurus* bulls.

Gonadotropin secretion during the infantile period is low due to reduced gonadotropin-releasing hormone (GnRH) secretion; maturation changes within the hypothalamus result in increased GnRH pulse secretion and drive the transition from the infantile period. Increased GnRH secretion is dependent on either the development of central stimulatory inputs or removal of inhibitory inputs. Hypothalamus weight and GnRH content do not increase during the infantile period, but hypothalamic concentrations of estradiol receptors decrease after one month of age [7]. However, the hypothesis that GnRH secretion is low during infancy due to elevated sensitivity of the hypothalamus to the negative feedback of sex steroids (gonadostat hypothesis) has been questioned in bulls, since castration does not alter luteinizing hormone (LH) pulse frequency or mean concentrations before two months of age [8]. Nonetheless, since GnRH secretion into hypophyseal portal blood is not necessarily accompanied by LH secretion during the infantile period, experiments that use LH concentrations to infer GnRH secretion patterns during this period need to be interpreted with caution [9]. Another possibility is that removal of opioidergic inhibition and/or increased dopaminergic activity may be involved in triggering the increase in GnRH secretion during the infantile period. Opioidergic inhibition of LH pulse frequency during the infantile period has been demonstrated by increased LH secretion between one and four months of age in bulls treated with naloxone, an opioid competitive receptor antagonist [10], whereas concentrations of norepinephrine, dopamine, and dopamine metabolites increased twofold to threefold in the anterior hypothalamic–preoptic area in bulls aged 0.5–2.5 months [11].

Direct evaluation of blood samples from the hypophyseal portal system has demonstrated that GnRH pulsatile secretion increases linearly from age two weeks (3.5 pulses per 10 hours) to 12 weeks (8.9 pulses per 10 hours) in bulls. Although GnRH secretion into hypophyseal portal blood was detected at two weeks, pulsatile LH secretion was not detected in jugular blood samples before eight weeks of age. In addition, GnRH pulses are not necessarily accompanied by LH secretion until 8–12 weeks of age, when all GnRH pulses result in LH pulses. The increase in pulsatile GnRH release from two to eight weeks of age without a concomitant increase in LH secretion may represent a reduced ability of the pituitary gland to respond to GnRH stimulus [9]. The period in which GnRH pulses do not stimulate LH secretion correspond to a period during which there is an increase in pituitary weight, GnRH receptor concentration, and LH content [7]. Moreover, frequent GnRH treatments during the infantile period in calves increases pituitary LH-β mRNA, LH content, and GnRH receptors, with resulting increases in LH pulse frequency and mean concentrations [12], indicating that increased

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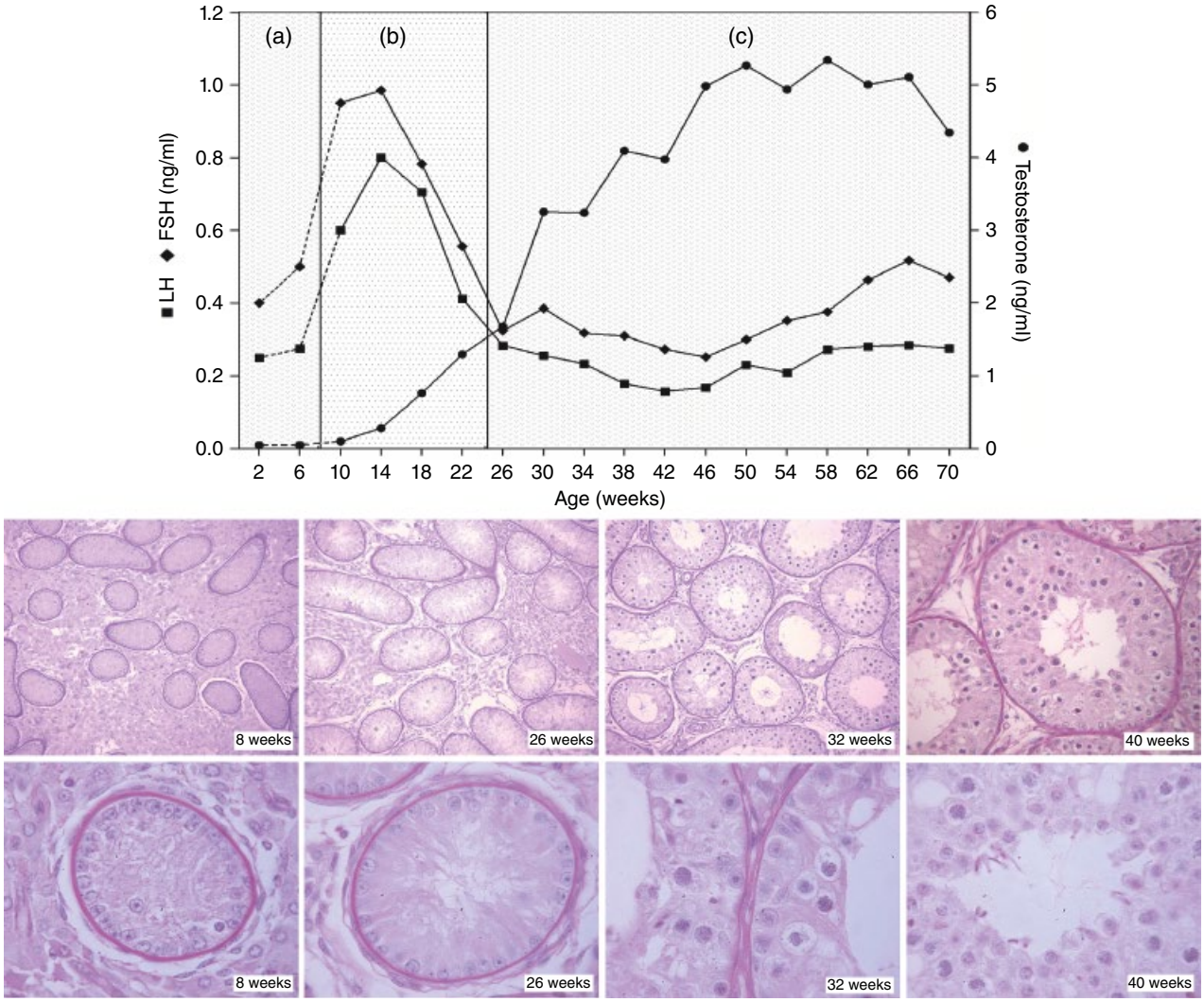


Figure 5.1 Mean serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone concentrations in bulls during the infantile (a), prepubertal (b), and pubertal/postpubertal (c) periods. Data from 2 to 6 weeks are adapted from Hereford × Charolais bulls [1]. Data from 10 to 70 weeks are from Angus and Angus × Charolais bulls receiving adequate nutrition [2–5]. Infancy is the period that extends from birth until approximately eight weeks of age. During this period gonadotropin and testosterone concentrations are low, the testicular parenchyma is occupied mostly by interstitial tissue, seminiferous cords are lined by undifferentiated Sertoli cells, and centrally located gonocytes with large nuclei can be observed. The prepubertal period is characterized by a dramatic increase in gonadotropin concentrations (the early gonadotropin rise) and by slowly increasing testosterone secretion. The prepubertal period extends from approximately 10 to 26 weeks of age and during this period a cord lumen begins to develop and gonocytes migrate toward the basement membrane, differentiating into spermatogonia. Gonadotropin concentrations decrease concomitantly with a rapid increase in testosterone concentration during the pubertal period, which also coincides with the start of a phase of rapid testicular growth. Formation of the tubular lumen is evidence of Sertoli cell differentiation and development of a functional blood–testis barrier that precedes the appearance of primary spermatocytes and spermatids around 32 weeks of age. With continuous increase in diameter, seminiferous tubules occupy most of the testicular parenchyma and start to produce mature sperm at approximately 40 weeks of age. *Sources:* Data from [2, 4–6].

GnRH pulse frequency results in increased pituitary sensitivity to GnRH. With time, the increased GnRH secretion results in the increased LH pulse frequency observed during the prepubertal period.

From birth until approximately two months of age, mesenchymal-like cells comprise the majority of the cells in the testicular interstitial tissue. Typical Leydig cells constitute about 6% of all intertubular cells at one month of age and a number of these cells are found in an advanced degenerative state, probably as remnants of the fetal Leydig cell population. Degenerating fetal and newly formed Leydig cells coexist until two months of age, but only Leydig cells formed postnatally are observed thereafter [13, 14]. The diameter of the seminiferous tubules is approximately

50 µm during the infantile period; tubule is actually a misnomer, since these are in fact solid cords with no lumen at this stage of development. Undifferentiated Sertoli cells (or undifferentiated supporting cells) are the predominant intratubular cells from birth until approximately four months of age. The number of undifferentiated Sertoli cells remains constant until one month of age, but cell multiplication is maximal between one and two months of age, decreasing thereafter until approximately four months of age. During the infantile period, the membranes of neighboring undifferentiated Sertoli cells contain few interdigitations and no special junctional complexes [15, 16]. These immature Sertoli cells produce and secrete anti-Müllerian hormone (AMH) and circulating concentrations increase until two months of age [1]. The germ cell population is composed solely of gonocytes (or prespermatogonia) at birth. Gonocytes are usually centrally located and have a large nucleus (~12 µm in diameter) with a well-developed nucleolus. Gonocyte proliferation slowly resumes between one and two months of age [15, 17].

Prepubertal Period

The prepubertal period is characterized by a temporary increase in gonadotropin secretion, the so-called early gonadotropin rise. The early gonadotropin rise is a critical event in the sexual development of bulls. Not only is it associated with dramatic changes in testicular cellular composition, initial increase in testosterone secretion, and timing of attainment of puberty, but also it has long-lasting effects on testicular growth and sperm production. This period extends from approximately two to six months of age in *B. taurus* bulls.

The early gonadotropin rise is driven by increased GnRH pulse secretion, as demonstrated by a dramatic increase in LH pulse frequency (Figure 5.2); pulsatile discharges of follicle-stimulating hormone (FSH) have been observed in bulls but are much less evident than those of LH. The number of LH pulses increases from less than one per day at one month to approximately 12–16 per day (one or more pulses every two hours) at approximately four months of age. Changes in pulse amplitude during this period are not consistent among reports; amplitude may be reduced, unchanged, or augmented [2, 7, 18–21]. LH-binding sites are present in testicular interstitial tissue at birth and at four months of age and pulsatile LH secretion is an essential requirement for Leydig cell proliferation, differentiation, and maintenance of fully cell differentiated structure and function [22]. Mesenchymal-like cells in the testes cease to proliferate around four months of age and start to differentiate into contractile myofibroblasts and Leydig cells. Differentiating, mitotic, and degenerating Leydig cells are observed in close proximity from four to seven months of age. Leydig cell numbers and mass per testis increase from one month (0.42 billion and 0.15 g/testis, respectively) to seven months of age (6 billion and 5.8 g/testis, respectively), but mitosis after this age is rare [13, 14]. Circulating insulin-like peptide 3 (INSL3), a major product and biomarker of Leydig cell functional capacity, increases linearly between two and six months in bulls [6, 23].

The characteristic pulsatile nature of LH secretion is important for testosterone production, since continuous exposure of Leydig cells to LH results in reduced steroidogenic responsiveness due to downregulation of LH receptors [24]. Initiation of Leydig cell steroidogenesis is characterized by increased androstenedione secretion, which decreases as the cells complete maturation and begin secreting testosterone. During the first three to four months of age, testosterone concentrations are low and secretion does not necessarily accompany LH pulses. After this age, LH pulses are followed by testosterone pulses and mean testosterone concentrations begin to increase. The number of testosterone pulses increases from 0.3–2.3 pulses per 24 hours at one to four months of age to 7.5–9 pulses per 24 hours at five months of age [25–28].

The crucial role of the LH secretion pattern during the early gonadotropin rise in regulating sexual development in bulls has been demonstrated in several studies using a variety of approaches. Prolonged treatment with a GnRH agonist in calves aged 1.5–3.5 months decreased LH pulse frequency, pulse amplitude, and mean concentrations at three months of age, delayed the peak mean LH concentration from five to six months of age, and reduced testosterone concentrations between 3.5 and 4.5 months of age. These hormonal alterations were associated with delayed puberty and reduced testes weight and number of germ cells in tubular cross-sections at 11.5 months of age. On the other hand, treatment with GnRH every two hours to mimic pulsatile secretion from 1 to 1.5–2 months of age increased LH pulse frequency and mean concentration during the treatment period and resulted in greater scrotal circumference, testes weight, seminiferous tubules diameter, and number of germ and Sertoli cells in tubular cross-sections at 12 months of age [29–31]. The LH secretion pattern during the prepubertal period is also associated with age at puberty in bulls raised in contemporary groups, suggesting that this is the physiological mechanism by which genetics affect sexual development. Studies have shown that LH pulse frequency was greater around 2.5–5 months of age and that mean LH concentrations increased earlier and reached greater maximum levels in early- than in late-maturing Hereford bulls (age at puberty 9.5 and 11 months, respectively) [19, 21].

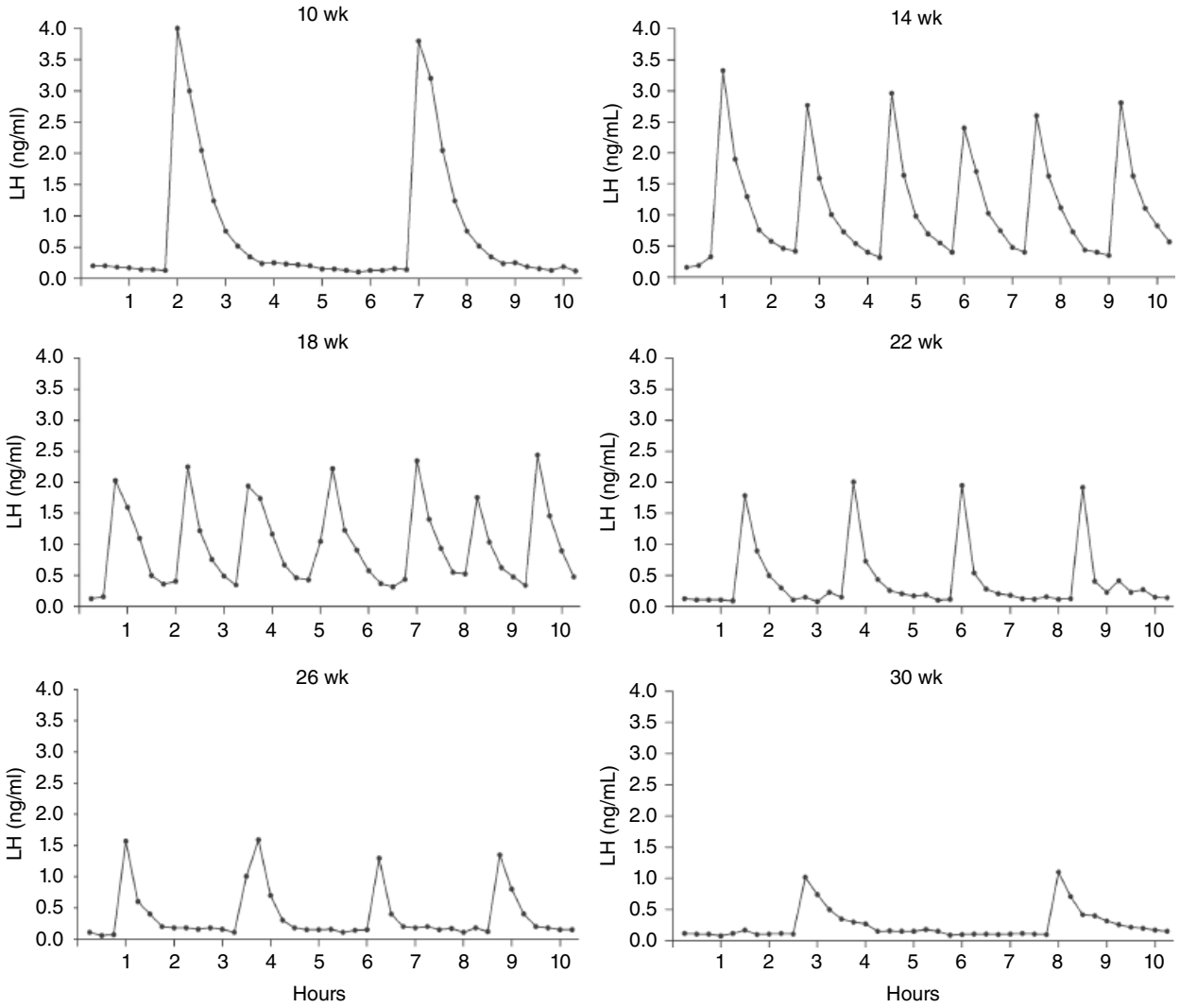


Figure 5.2 Serum LH concentrations between 10 and 30 weeks of age in Angus and Angus × Charolais bulls. Graphs closely exemplify the mean pulse frequency and pulse amplitude observed in bulls receiving adequate nutrition. *Sources:* [2, 4, 6].

Additional support for the crucial role that LH secretion pattern during the early gonadotropin rise plays in sexual development in bulls has been provided by studies demonstrating that nutrition during the prepubertal period affects LH secretion pattern, age at puberty, and testicular development. In studies with beef and dairy bulls receiving different nutrition from 2 to 16 months of age, reduced LH pulse frequency during the prepubertal period resulted in delayed puberty in bulls receiving low nutrition, while a more sustained increase in LH pulse frequency in bulls receiving high nutrition was associated with hastened testosterone production and greater testes weight at 16–19 months of age when compared with bulls receiving low and medium (control) nutrition (results in beef bulls are shown in Figure 5.3). These observations were corroborated by additional studies designed to investigate the effects of nutrition specifically during the prepubertal period. Bulls that received high nutrition only from two to seven months of age also had a more sustained increase in LH pulse frequency, had greater testosterone secretion, were approximately two to four weeks younger at puberty, and had greater testes weight at 16–19 months of age when compared with bulls receiving control nutrition (results in beef bulls are shown in Figure 5.4). On the other hand, reduced LH secretion resulting from low nutrition from two to six months of age was associated with increased age at puberty and smaller testes weight at 16–19 months of age even when these bulls received control or high nutrition after seven months of age and LH and testosterone secretion were

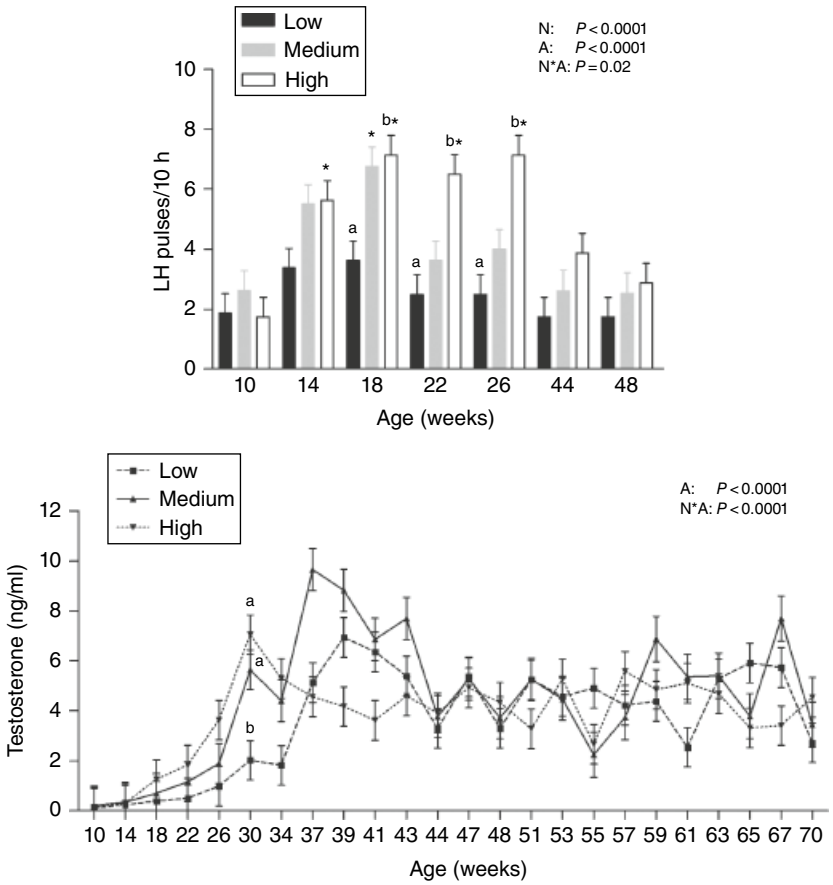


Figure 5.3 Mean (± SEM) number of LH pulses and serum testosterone concentrations in Angus and Angus × Charolais bulls receiving low, medium (control), or high nutrition from 10 to 70 weeks of age. N, A, and N*A indicate nutrition, age, and nutrition-by-age interaction effects, respectively. Superscripts indicate differences ($P < 0.05$): a,b, group differences within age; *, age differences within group. Bulls in the low nutrition group were older at puberty (321 days) than bulls in the medium and high nutrition groups (299 and 288 days, respectively), whereas bulls in the high nutrition group had greater paired-testes weight at 70 weeks of age (655 g) than bulls in the low and medium nutrition groups (520 and 549 g). *Source*: From [2], © 2007, Elsevier.

not different after the change in nutrition (results in beef bulls are shown in Figure 5.5) [2–4, 32–35].

During the prepubertal period there is a progressive increase in the proportion of testicular parenchyma occupied by seminiferous tubules, and seminiferous tubule diameter increases to approximately 125 µm at six months of age [20, 36]. FSH-binding sites can be observed in seminiferous tubules of bull calves at birth and at four months age, and decreased inhibin concentrations, coupled with increased FSH concentrations, stimulate the proliferation of undifferentiated Sertoli cells [6, 22]. Although there is considerable evidence that FSH is essential for normal Sertoli cell function, the period of Sertoli cell differentiation coincides with the initiation of testosterone secretion by the Leydig cells, indicating that testosterone may also be involved in promoting maturation of undifferentiated Sertoli cells. At approximately four months of age, undifferentiated Sertoli cells enter the G_0 phase of the cell cycle for the rest of the bull's life. With the end of the proliferative phase, undifferentiated Sertoli cells begin to transform into adult-type Sertoli cells. Opposing cell membranes of adjacent undifferentiated Sertoli cells start to develop extended junctional complexes above the spermatogonia and in the basal portion of the tubules; "cracking" of the tubular cytoplasm is first detected around six months of age [1, 15–17]. As immature Sertoli cells begin to differentiate they cease to secrete AMH and circulating concentrations of this hormone decrease after four months of age [1]. FSH secretion, maturation of Sertoli cells, and increased testosterone secretion are probably also involved in the differentiation of gonocytes into spermatogonia. Gonocytes are gradually displaced to a position close to the basal lamina and divide by mitosis, originating A-spermatogonia. Differentiation and degeneration result in the complete disappearance of gonocytes from the seminiferous tubules by five months of age. A-spermatogonia divide mitotically

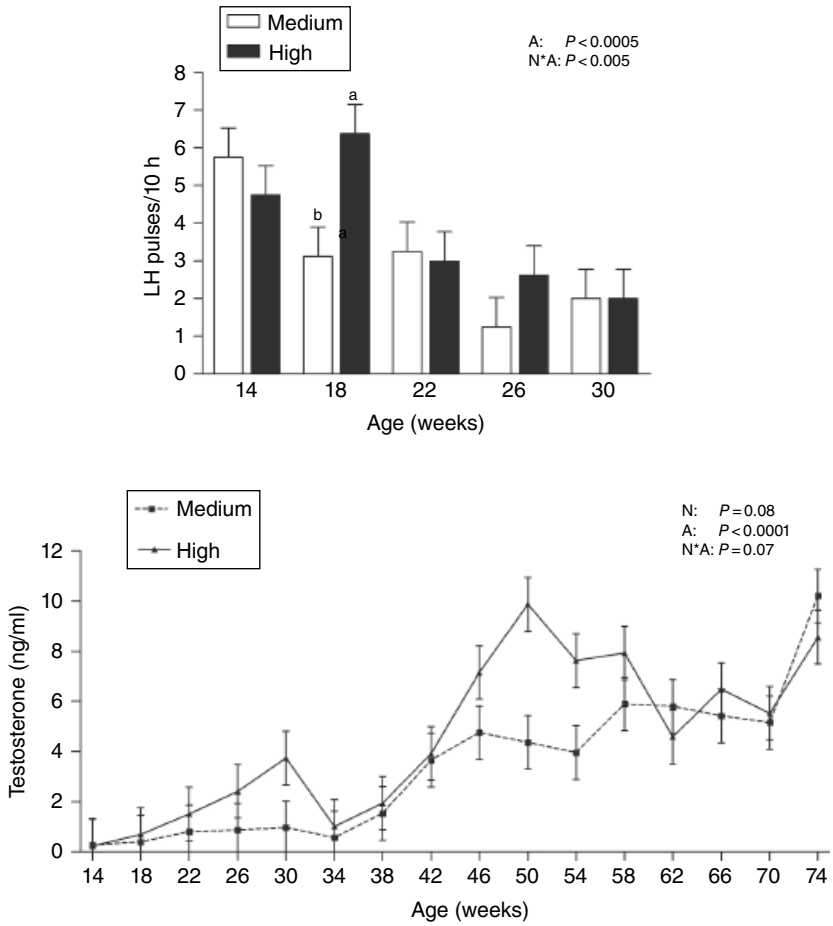


Figure 5.4 Mean (± SEM) number of LH pulses and serum testosterone concentrations in Angus and Angus × Charolais bulls receiving medium (control) or high nutrition from 10 to 30 weeks of age and the same medium nutrition from 31 to 74 weeks. N, A, and N*A indicate nutrition, age, and nutrition-by-age interaction effects, respectively. Superscript a and b indicate differences ($P = 0.09$) between groups within age. Bulls in the high nutrition group were younger at puberty (314 days) and had greater paired-testes weight at 74 weeks of age (610 g) than bulls in the medium group (327 days and 531 g, respectively). *Source*: From [3], © 2007, Elsevier.

to form In-spermatogonia and B-spermatogonia, which in turn enter meiosis around four to five months of age [15, 17, 20, 36, 37].

Pubertal Period

The pubertal period is characterized by reduced gonadotropin secretion, increased testosterone secretion, initiation of spermatogenesis, and the eventual appearance of sperm in the ejaculate. This period also coincides with the start of a phase of rapid testicular growth (see Chapter 6 for testicular growth charts) and extends from approximately 6 to 12 months of age in *B. taurus* bulls.

The rapidly increasing testosterone secretion and possibly increased hypothalamic sensitivity to negative feedback from androgens are likely responsible for the decrease in LH secretion during the pubertal period. Although immunization with inhibin antiserum results in a marked increase in FSH concentrations in prepubertal bulls, whether inhibin produced by Sertoli cells acts on the gonadotrophs to limit FSH secretion is uncertain, since circulating inhibin decreases steadily from birth to seven months of age [6, 38, 39]. After seven months of age, Leydig cell mass increases slowly but continuously to reach about 10 g in the young adult testis at 24 months of age as a result of considerable increase in Leydig cell volume (hypertrophy); Leydig cell mitochondrial mass more than doubles from 10 to 24 months of age [14]. Testosterone pulse frequency does not increase after the peripubertal period and remains at approximately 4.5–6.8 pulses per 24 hours from 6 to 10 months of age. However, pulse amplitude increases during the pubertal period with consequent increase in testosterone mean concentrations until approximately 12 months of age. Elevated testosterone secretion is essential for initiation of spermatogenesis [12, 25–27, 40].

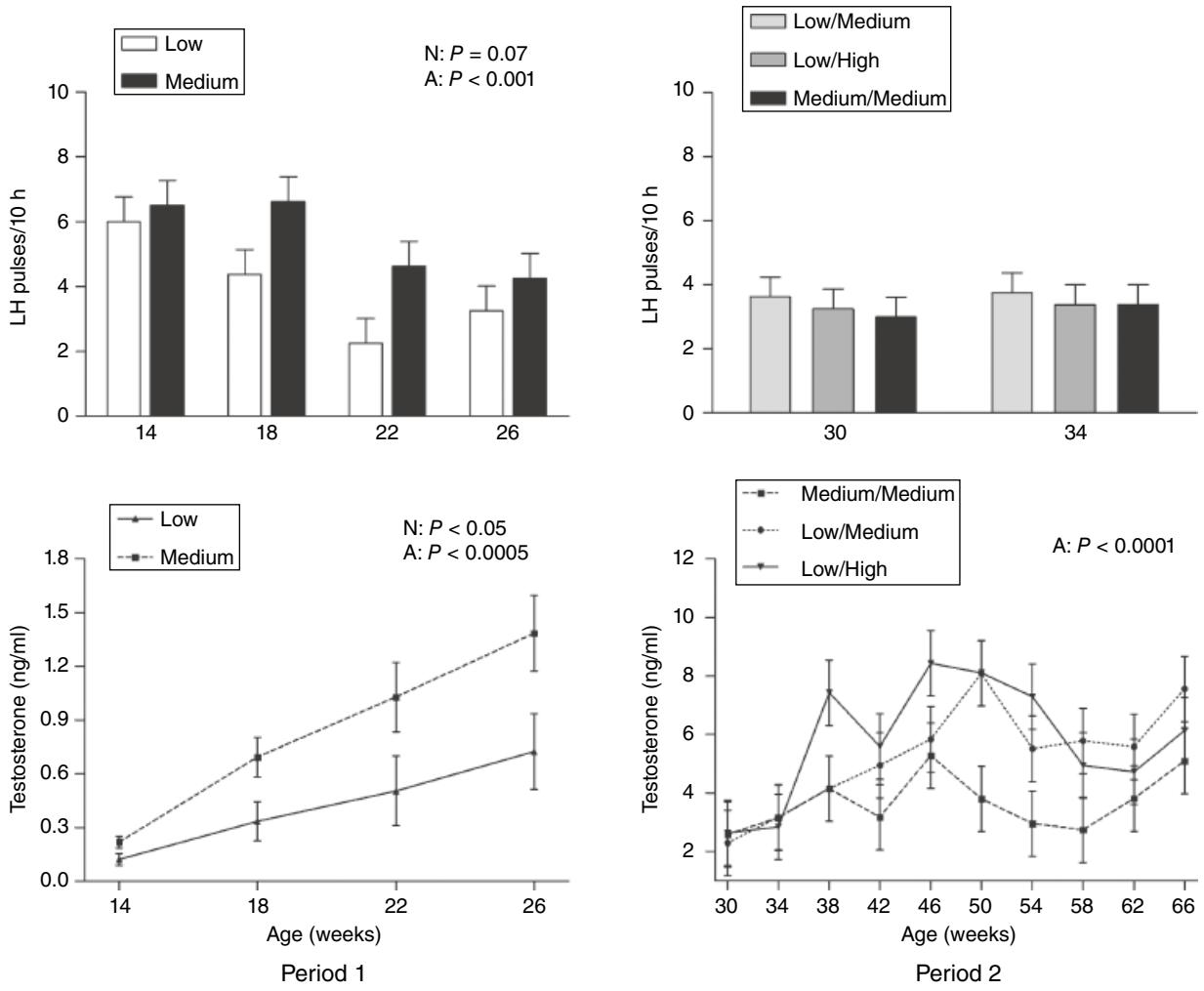


Figure 5.5 Mean (± SEM) number of LH pulses and serum testosterone concentrations in Angus and Angus × Charolais bulls receiving medium (control) from 10 to 70 weeks of age or low nutrition from 10 to 26 weeks of age and either medium or high nutrition from 27 to 70 weeks of age. N, A, and N*A indicate nutrition, age, and nutrition-by-age interaction effects, respectively. Bulls in the medium/medium nutrition group were younger at puberty and had greater paired-testes weight at 70 weeks of age than those in the low/medium nutrition group (293 vs 331 days and 600 vs 528 g, respectively). Age at puberty and paired-testes weight for bulls in the low/high nutrition group were intermediate (313 days and 553 g, respectively). *Source*: From [4], © 2007, Society for Reproduction and Fertility.

Seminiferous tubule diameter increases to approximately 200 µm at eight months of age and reaches 240 µm by 16 months [20, 36, 41]. Total seminiferous tubule length increases from 830 m per testis at three months of age to 2010 m per testis at eight months of age in Holstein bulls [36]. Most Sertoli cells complete their morphological differentiation and attain adult structure after six to seven months of age. Junctional complexes consisting of many serially arranged points or lines of fusion involving neighboring Sertoli cell membranes can be observed. These junctions form a functional blood–testis barrier and divide the tubular epithelium into a basal compartment containing spermatogonia and an adluminal compartment containing germ cells at later stages of spermatogenesis; formation of a functional blood–testis barrier is accompanied by formation of the tubular lumen and precedes the appearance of primary spermatocytes and more advanced germ cells [1, 15–17]. In Holstein bulls, the number of adult-type Sertoli cells increases dramatically from 202 to 8862 million cells per testis between five and eight months of age, respectively [36].

Germ cell proliferation is maximal between four and eight months of age and represents the expansion of the spermatogonial stem cell. In Holstein bulls, the number of spermatogonia increases from 181 million cells per testis at four months of age to 3773 million cells per testis at eight months of age; the number of spermatogonia continues to increase until approximately 12 months of age [36]. Primary spermatocyte numbers increase slowly until eight months

of age, when the numbers exceed the number of spermatogonia. Secondary spermatocytes and round spermatids first appear at approximately six to seven months of age, whereas elongated spermatids appear around eight months of age. The number of spermatids increases rapidly after 10 months of age when spermatid numbers exceed the numbers of any other germ cell. Mature sperm appear in the seminiferous tubules at approximately 8–10 months of age. Testes weighing more than 100 g in Swedish Red-and-White bulls or more than 80 g in Holstein bulls are likely to be producing sperm [15, 17, 20, 36, 37]. Spermatogenesis eventually reaches a level of efficiency (i.e. increasing number of more advanced germ cells resulting from individual precursor cells) that results in the production of a number of sperm sufficient for those to appear in the ejaculate, the physiological event that characterizes puberty (see Chapter 6).

Metabolic Hormones During the Prepubertal and Pubertal Periods

The mechanisms controlling reproduction and energy balance are intrinsically related and have evolved to confer reproductive advantages and guarantee the survival of species. The neural apparatus designed to gauge metabolic rate and energy balance has been denominated the body "metabolic sensor." This sensor translates signals provided by circulating (peripheral) concentrations of specific hormones into neuronal signals that ultimately regulate the GnRH pulse generator and control the reproductive process. Metabolic indicator hormones may serve as signs to the hypothalamus–pituitary–gonadal axis and affect sexual development. The patterns of some of these hormones have been studied in growing beef bulls (Figure 5.6). In contrast to species in which circulating growth hormone (GH) concentrations continue to increase until after puberty, GH concentrations decrease during the pubertal period in bulls [2, 5]. Differences in the stage of body development at which each species attains puberty are likely responsible for the different GH profiles among species. Accordingly, the GH profile in bulls seems to indicate that a relatively advanced stage of body development must be attained before the gonads are efficiently producing sperm. The differences in GH secretion among species may be due to the regulatory role of steroids on GH secretion. In other species, steroids stimulate GH secretion, but GH concentrations do not differ between intact bulls and castrated steers [42]. Moreover, decreasing GH concentrations during sexual development are observed along with increasing testosterone concentrations, indicating that steroids do not have a positive feedback on GH secretion in bulls as in other species [2, 5].

Circulating insulin-like growth factor (IGF)-I concentrations in bulls increase continuously and only reach a plateau (or decrease slightly) after sexual development is mostly completed after 12–14 months of age; increasing circulating concentrations of IGF-binding protein 3 and decreasing concentrations of IGF-binding protein 2 are also observed during sexual development [2–5, 43–45]. The concomitant decrease in circulating GH concentrations with the increase in IGF-I concentrations during sexual

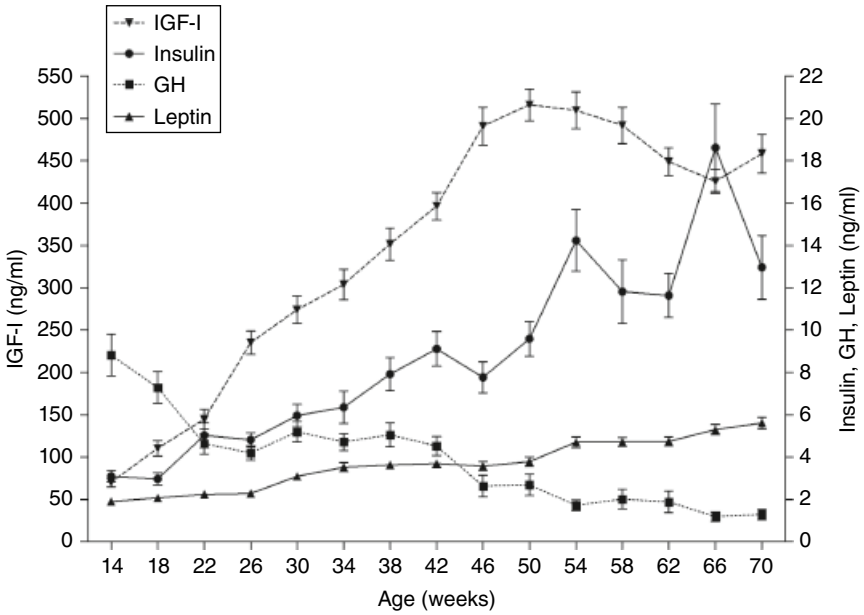


Figure 5.6 Mean (± SEM) serum IGF-I, insulin, GH, and leptin concentrations during sexual development in Angus and Angus × Charolais bulls receiving adequate nutrition. *Sources:* Data from [2–5].

development in bulls indicates that either there are drastic changes in liver sensitivity to GH or other sources are responsible for IGF-I production. A possible IGF-I source might be the testes, since Leydig cells are capable of secreting this hormone in other species. Observations that intact bulls tend to have greater IGF-I concentrations than castrated steers at 12 months of age further support the hypothesis that the testes might contribute substantially to circulating IGF-I concentrations during the prepubertal and pubertal periods in bulls [42]. Close temporal associations observed in a series of nutrition studies strongly suggest that circulating IGF-I might be involved in regulating the GnRH pulse generator and the magnitude and duration of the early gonadotropin rise in beef bulls [2–4, 33].

A possible effect of IGF-I on testicular steroidogenesis in bulls has also been suggested. Leydig and Sertoli cells produce IGF-I, indicating the existence of paracrine/autocrine mechanisms of testicular regulation involving IGF-I [46, 47]. It is assumed that most of the IGF-I in the testes is produced locally and that circulating IGF-I may play a secondary role in regulating testicular development and function. However, the temporal patterns and strong associations among circulating IGF-I concentrations, testicular size, and testosterone secretion observed in bulls receiving different nutrition argue for a primary role for this hormone [2–4, 33, 35]. The primary role of increased circulating IGF-I during the pubertal period may be to promote the increase in testosterone concentrations by regulating Leydig cell multiplication, differentiation, and maturation. Since testosterone upregulates IGF-I production and IGF-I receptor expression by Leydig and Sertoli cells [48], the establishment of a positive feedback loop between IGF-I secretion and testosterone production may be important for sexual development.

Circulating leptin and insulin concentrations also increase during the pubertal period in bulls. However, developmental and nutritional differences in LH pulse frequency are not related to differences in leptin or insulin concentrations in beef or dairy bulls [2, 3, 35]. Other studies have also demonstrated that leptin does not stimulate *in vitro* GnRH secretion from hypothalamic explants or gonadotropin secretion from adenohypophyseal cells collected from bulls and steers maintained at an adequate level of nutrition [49]. These results indicate that the role of these hormones in regulating GnRH secretion, if any, might be purely permissive in bulls.

GnRH-Independent Testicular Development

The rapid testicular growth observed after six months of age in bulls occurs when circulating gonadotropin concentrations are decreasing, which points to the existence of important GnRH-independent mechanisms regulating testicular development. The period of accelerated testicular growth coincides with increasing circulating IGF-I and leptin concentrations, and strong associations between these hormones and testicular size have been observed in growing beef and dairy bulls [2, 5, 6, 33], indicating that metabolic hormones may be involved in regulating GnRH-independent testicular development. Since there was no association between circulating metabolic hormones and gonadotropin concentrations in these studies, the possible effects of metabolic hormones on testicular growth are likely direct and independent of the hypothalamus and pituitary. Accelerated testicular growth in bulls involves increases in seminiferous tubule diameter and length, volume of testicular parenchyma occupied by seminiferous tubules, and total number of germinal cells [15, 36]. Although IGF-I and leptin concentrations are associated with testicular size, there are no associations between these hormones and seminiferous tubule diameter and area, seminiferous epithelium area, or volume occupied by seminiferous tubule (L.F.C. Brito, unpublished results). These observations suggest that increased circulating IGF-I and leptin concentrations are associated with increased length of the seminiferous tubules and likely with overall increases in the total number of testicular cells. Considering the cellular events in the testis during the pubertal period, the temporal patterns of metabolic hormone concentrations indicate that circulating IGF-I and leptin could be involved in regulating Leydig cell multiplication and maturation, Sertoli cell maturation, and germ cell multiplication during the period of accelerated GnRH-independent testicular growth in bulls.

Testicular concentrations of LH and FSH receptors in beef bulls decrease around five to six months of age, but increase thereafter until at least approximately 13 months of age, which might increase the sensitivity of Leydig and Sertoli cells to the low concentrations of gonadotropins during the rapid testicular growth phase [37]. Other mechanisms that might be associated with GnRH-independent testicular growth include changes in testicular concentrations and bioavailability of growth factors such as transforming growth factor (TGF)-α and TGF-β1, TGF-β2, and TGF-β3 and interleukin (IL)-1α, IL-1β, and IL-6 [50, 51]. In addition, experiments evaluating the effect of nutrition on sexual development have demonstrated that the impact of gonadotropins on target tissues during the prepubertal period has long-term effects on testicular development in bulls. Bulls with either greater LH pulse frequency or more sustained increase in LH pulse frequency during the early gonadotropin rise had a more prolonged period of increased testicular growth and greater testicular size at 15–16 months of age, even when no differences in metabolic hormones or testosterone concentrations were observed after six months of age. These results indicate that the putative effects of circulating metabolic

hormones, gonadotropins, local growth factors, and other unknown factors during the period of rapid testicular growth might be dependent on previous LH exposure during the prepubertal period. The LH secretion pattern during the early gonadotropin rise seems to "prime" testicular development and dictates maximum testicular size in bulls [2–4, 33].

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6

Sexual Development and Puberty in Bulls

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Introduction

Age at puberty is a major determinant of cattle production efficiency. The ability to breed animals at younger ages reduces generation intervals and increases genetic gains. However, reduced sperm production and poor semen quality due to immaturity are common causes of poor reproductive performance of young bulls and represent a serious loss of superior genetic stock. The ability to collect and freeze semen from younger bulls is also desired to reduce the time required for progeny testing and to accelerate the process of artificial insemination and sire selection. Therefore an understanding of pubertal changes and the factors that affect sexual development is required in order to promote the successful use of young bulls for reproductive purposes.

Testicular Development

Sexual development is associated with marked gonadal growth. Scrotal circumference (SC) is highly correlated with testicular weight (Figure 6.1) and is the most common endpoint evaluated to determine testicular development. The testicular growth curve in bulls shows an initial period of little growth followed by a rapid growth phase and then a plateau (Figures 6.2 and 6.3). Although the overall pattern of testicular growth is somewhat similar in all breeds, the characteristics of the growth curve are greatly affected by genetics. In general, the rapid growth phase is shorter and testicular growth plateaus sooner in bulls from breeds that mature faster (reach puberty earlier) than in bulls from late-maturing breeds, resulting in marked differences in the curve slope. This is especially evident when *Bos taurus* bulls are compared with *Bos indicus* bulls, which in general reach puberty later than the former. The asymptotic value of the testicular growth curve, namely adult testicular size, also differs considerably among breeds (Figures 6.2–6.6) [1–8]. These same differences can be observed within breeds between early- and late-maturing bulls (Figure 6.7), emphasizing the effects of genetics on testicular growth [9–11].

SC is a moderately heritable trait in cattle; yearling heritability estimates are 0.36–0.55 in Angus [12–16], 0.28 in Brahman [17], 0.40–0.71 in Hereford [14, 15, 18–24], 0.67 in Holstein [25], 0.46 in Limousin [26], 0.39–0.60 in Nelore [27–30], 0.32 in Red Angus [31], and 0.48 in Simmental bulls [14]. Therefore direct selection can have a very significant impact on SC. For example, selection of Santa Gertrudis bulls based on minimum SC over a 10-year period resulted in significant changes in average SC in one herd [32], whereas testicular weight at weaning was greater in the progeny sired by Limousin bulls with high expected progeny difference (EPD) for SC compared with progeny sired by bulls with average or low EPD [33]. Results from the Swedish performing station involving beef bulls of various breeds revealed an increase in SC of approximately 1 cm between 1997 and 2010 [34]. Similarly, comparison of data obtained from Canadian veterinary practices and testing stations between 2008 and 2011 from various beef breeds, with data compiled from the literature between 1972 and 1998, revealed that mean SC increased 0.8–2 cm in yearling bulls and 0.4–3.5 cm in two-year-old bulls [35]. Several studies have also demonstrated moderate to high phenotypic correlations between SC and growth traits and estimates of the genetic correlations with growth traits are generally positive (Table 6.1). Therefore the combination of direct selection for SC and indirect selection for growth traits is likely responsible for the general trend of increasing SC over the years in several breeds (Figure 6.4).

Heritability estimates for SC vary according to age. Studies have demonstrated that heritability estimates increase with age until approximately one year of age

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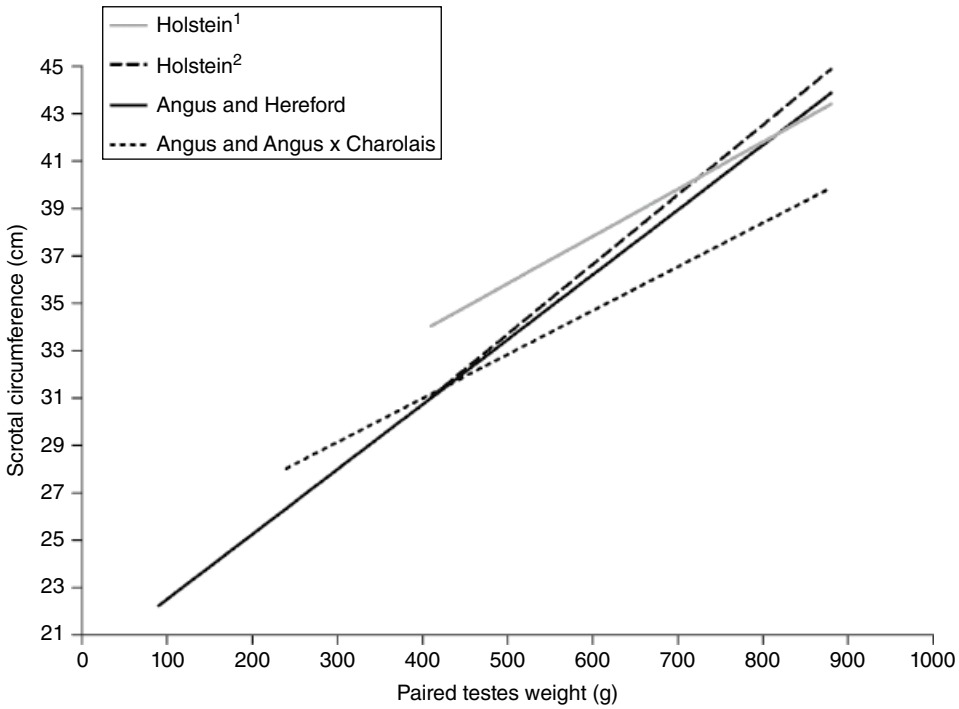


Figure 6.1 Regression lines for paired testes weight (PTW) according to scrotal circumference (SC). Holstein[1] measurements obtained from mature Holstein bulls (n = 35); PTW = −1298.5 + (50.2 × SC) [116]. Holstein[2] measurements obtained from Holstein bulls (n = 47) 19 to 184 months old; PTW = −654.4 + (34 × SC) [117]. Angus and Hereford SC measurements obtained from Hereford (n = 199) and Angus (n = 136) bulls 11 to 30 months old; PTW = −722.28 + (36.53 × SC) [3]. Angus and Angus × Charolais SC measurements obtained from Angus and Angus × Charolais bulls (n = 111) 14–16 months-old; PTW = −1274 + (54.04 × SC). *Source:* L. Brito, unpublished results.

(or 15–18 months of age in *B. indicus* bulls), whereas estimates for two-year-old bulls are lower [4, 15, 18, 25, 27, 28, 30, 36]. Therefore selection based on yearling SC is recommended over selection based on measurements obtained at other ages. Yearling SC is commonly recorded in performance evaluation programs for beef bulls, but age effects are very pronounced around this age since testicular growth is rapid during this stage of development (Figure 6.5). In order to adjust SC measurements to age 365 days, the Beef Improvement Program recommends use of the adjustment factors described in Table 6.2. Correlation coefficients between SC at one year of age and SC and paired testes weight at two years of age in Angus and Hereford bulls were 0.76 and 0.65, respectively, demonstrating that a bull with relatively small or large testes as a yearling will generally have comparable testes size as a two year old [3].

Attempts to establish guidelines for selection of bulls at weaning based on the likelihood of attainment of certain minimum yearling SC have produced mixed results. In one study, it was recommended that the minimum SC in Angus and Simmental bulls 198–291 days old should be 23 or 25 cm to ensure an SC of 30 or 32 cm at 365 days of age, respectively; the same recommendations for Hereford bulls were 26 and 28 cm [7]. In another study, differences between bulls that attained a minimum yearling SC of 34 cm and bulls that did not were observed for adjusted SC at 200 days of age (23.3 vs 20.5 cm, respectively). Based on these results, it was suggested that SC at weaning could be used to select bulls for breeding and 23 cm was proposed as the minimum SC standard at 200 days [37]. However, this study included bulls from several breeds with known differences in patterns of testicular growth and mature size, while using a singular and very strict yearling SC minimum. SC at 240 days of age could be used as a tool to select bulls with a high probability of meeting the minimum requirements for SC at 365 days of age (i.e. Simmental 32 cm; Angus, Charolais, and Red Poll 31 cm; Hereford 30 cm; Limousin 29 cm); sensitivity and specificity analysis for determining cutoff values indicated that the probability of Charolais bulls with SC ≥24 cm, Simmental and Limousin bulls with SC ≥22 cm, and Angus, Hereford, and Red Poll bulls with SC ≥21 cm attaining minimum requirements was greater than 80%. However, SC at weaning was not useful as a culling tool, since a large portion of bulls, irrespective of breed, met the minimum requirements at 365 days of age even when SC was below 21 cm at 240 days of age [38].

Although the heritability of semen traits is generally low, SC is positively associated with sperm production and

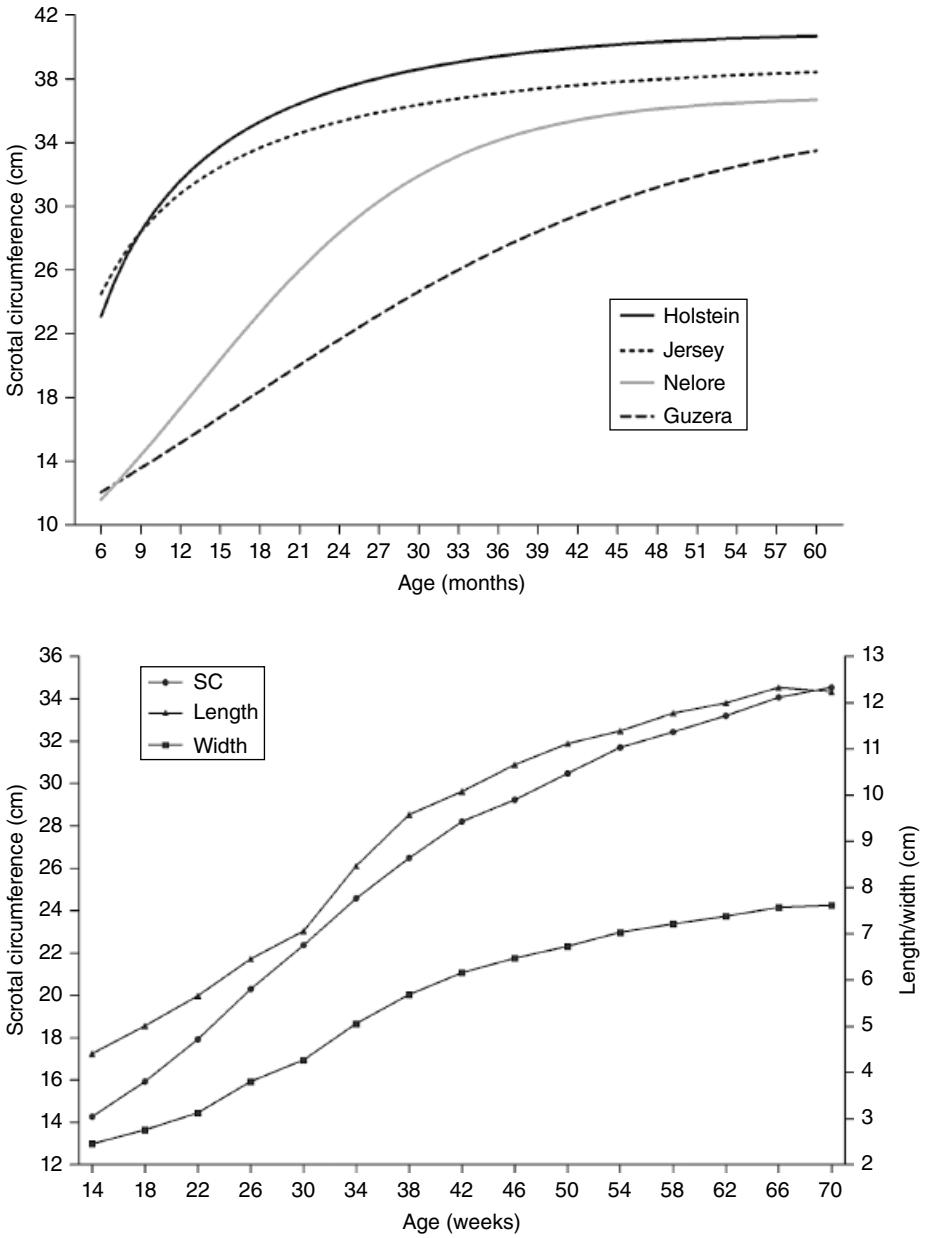


Figure 6.2 *Top*: regression curves for scrotal circumference (SC) according to age in Holstein, Jersey, Nelore, and Guzera bulls. Holstein measurements (n = 9614) obtained from bulls 6 to 77 months old; SC = −11.75 + [56.7 × (log AGE)] − [15.3 × (log AGE)^2]. *Source:* ASB Global Inc., unpublished results. Jersey measurements (n = 1038) obtained from bulls 7 to 75 months old; SC = −0.6814 + [40.26 × (log AGE)] − [10.27 × (log AGE)^2] (ABS Global Inc., unpublished results). Nelore measurements obtained from bulls (n = 532) 7 to 43 months old; SC = 36.9/1 + [4.22$^{-(0.11 \times \text{age})}$] [118]. Guzera measurements (n = 7410) obtained from bulls 2 to 69 months old; SC = 35.96/1 + [2.86$^{-(0.002 \times \text{age in days})}$] [119]. *Bottom*: testicular length and width associations with SC between 3 and 16 months of age in Angus and Angus × Charolais bulls (n = 111) receiving adequate nutrition. *Source:* L. Brito, unpublished results.

semen quality, and genetic correlations between SC and semen traits are generally favorable (Table 6.1). This suggests that direct selection for SC would be more effective in bringing about sperm production and semen quality improvement than direct selection pressure on semen traits themselves. In addition, several studies have reported an association between sire SC and daughter puberty. In Brahman and Hereford cattle, genetic correlations between SC and heifer ages at first detected ovulatory estrus, first breeding, and first calving were −0.32, −0.39, and −0.38, respectively [17, 39]. In another study with beef cattle, favorable relationships between greater sire SC and ages at puberty and at first calving were demonstrated by negative correlation coefficients between the two traits [40]. In a

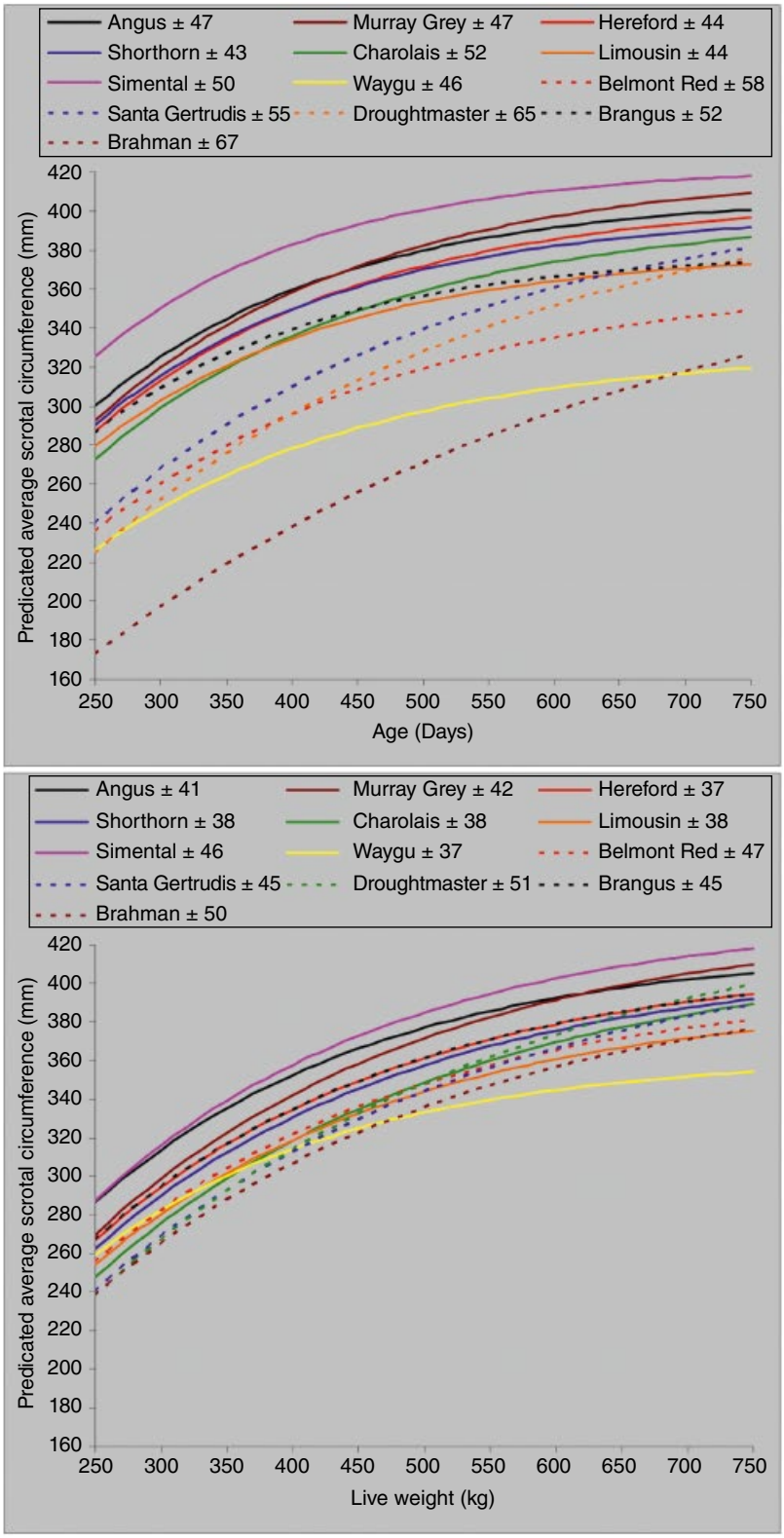


Figure 6.3 Scrotal circumference (± 90% confidence interval) in beef bulls of various breeds (*n* = approximately 300 000) according to age (*top*) and weight (*bottom*). *Source:* From [8], © 2014, Elsevier.

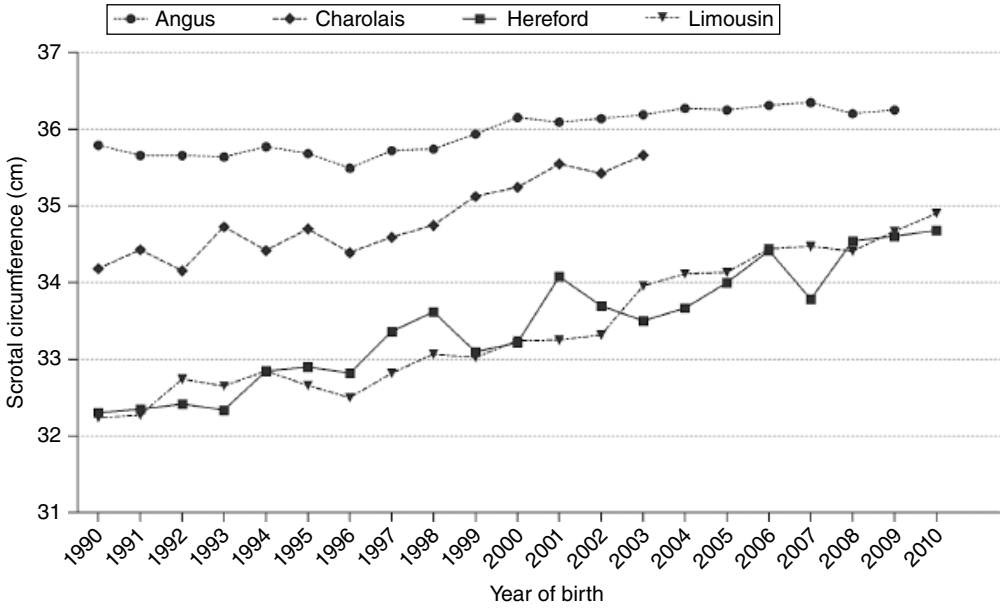


Figure 6.4 Mean scrotal circumference (SC) in registered yearling bulls according to year of birth. Measurements for Angus (unspecified number of bulls) and Limousin (n = 73 757; 1184–5200/year) bulls are adjusted to 365 days of age. Sources: North American Limousin Foundation; American Angus Association. Measurements for Charolais (n = 6984; 121–997/year) and Hereford (n = 5553; 360–536/year) bulls are unadjusted measurements obtained between 321 and 421 days of age. *Sources:* Canadian Hereford Association; Canadian Charolais Association.

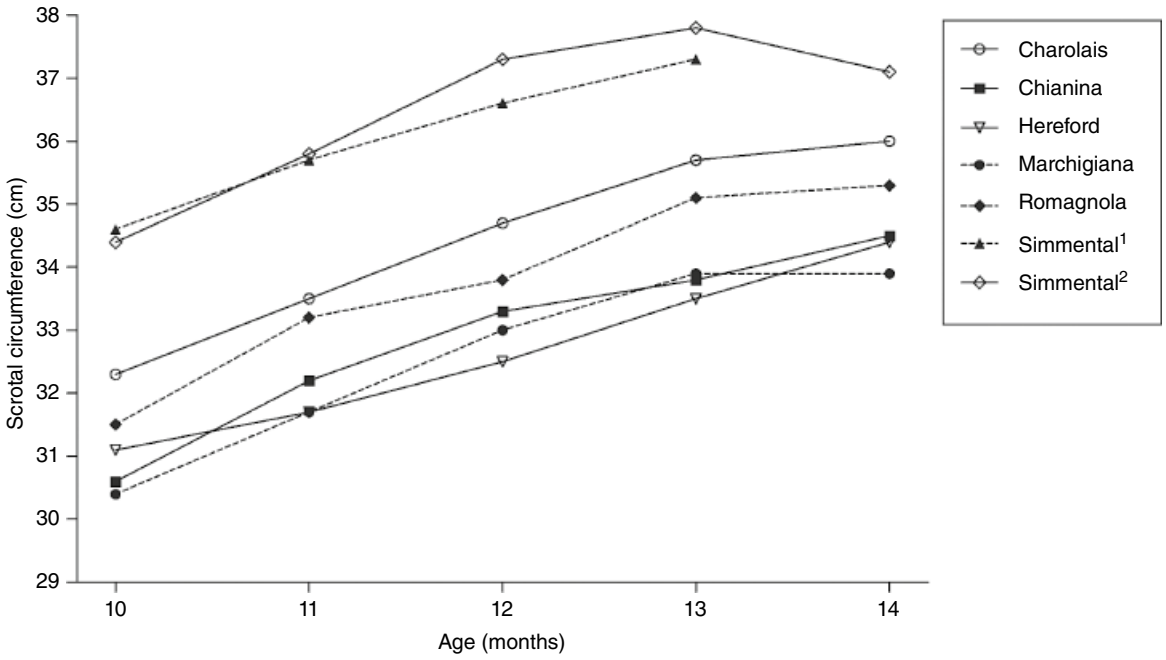


Figure 6.5 Mean scrotal circumference (SC) in yearling bulls. Charolais: n = 246–2622/age (source: Canadian Charolais Association). Chianina, Marchigiana, and Romagnola: n = 455, 415, and 425/age, respectively [118]. Hereford: n = 77–2510/age. *Source:* Canadian Hereford Association. Holstein: n = 162–1004/age. *Source:* ABS Global Inc. Simmental[1]: n = 129 to 2276/age. *Source:* Canadian Simmental Association. Simmental[2]: n = 120 to 2022/age. *Source:* American Simmental Association. Standard deviations are all between 2 and 3 cm. *Sources:* [1, 2, 118].

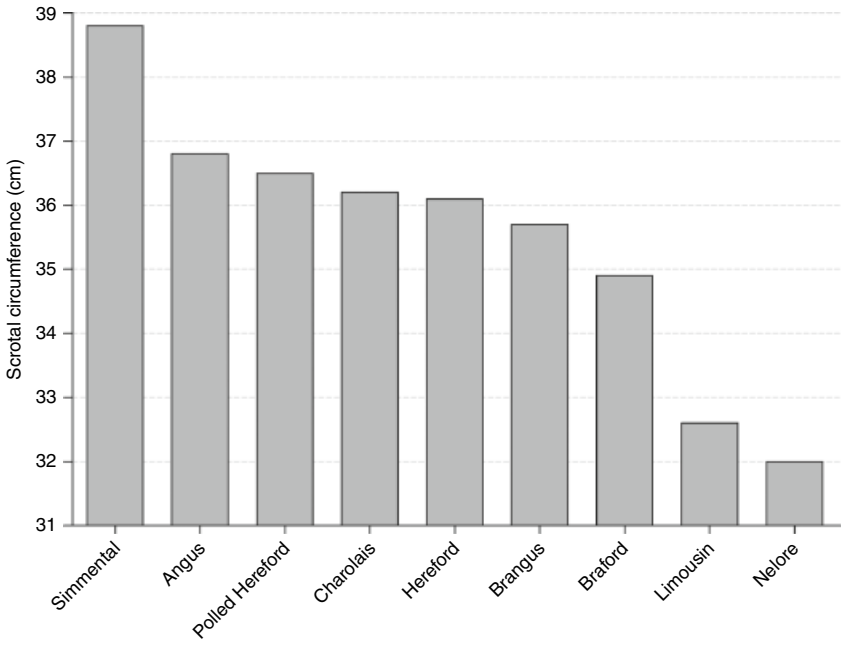


Figure 6.6 Weighted mean scrotal circumference (SC) reported for two-year-old bulls of various breeds: Simmental (n = 763), Angus (including Red Angus; n = 5046), Charolais (n = 1286), Hereford (including horned and polled; n = 8183), Brangus (n = 1312), Shorthorn (n = 271), Braford (n = 1210), Limousin (n = 229), Nelore (n = 6464). *Sources:* [4, 35, 119–121].

Table 6.1 Genetic correlations (r_g) between scrotal circumference, growth traits, and sperm production in bulls.

Breed	Growth trait	r_g	References
Angus	Yearling weight	0.24—0.68	[13, 15]
	Sperm concentration	0.54	[16]
	Sperm motility	0.36	
	Total sperm defects	−0.23	
Composite	Yearling weight	0.40—0.43	[36]
Hereford	Weaning weight	0.08—0.86	[15, 18]
	Yearling weight	0.30—0.52	[19–21]
	Weaning–yearling ADG	0.22—0.35	[22, 24]
	Sperm concentration	0.77	
	Sperm motility	0.34	
	Normal sperm	0.33	
Hereford/ Simmental	Sperm concentration	0.2	[14]
	Sperm motility	0.11	
	Total sperm number	0.19	
Limousin	Weaning weight	0.14	[26]
Nelore	Weaning weight	0.36	[27, 28]
	Yearling weight	0.34	
	Longissimus muscle area	0.28	
	Backfat thickness	0.17	
Red Angus	Yearling intramuscular fat	0.05	[31]
	Yearling carcass marbling score	0.01	
Various breeds	Birth–weaning ADG	0.02	[92, 122]
	Yearling weight	0.10—0.63	
	Weaning weight	0.56	
	Weaning–yearling ADG	0.59	

ADG, Average daily gain.

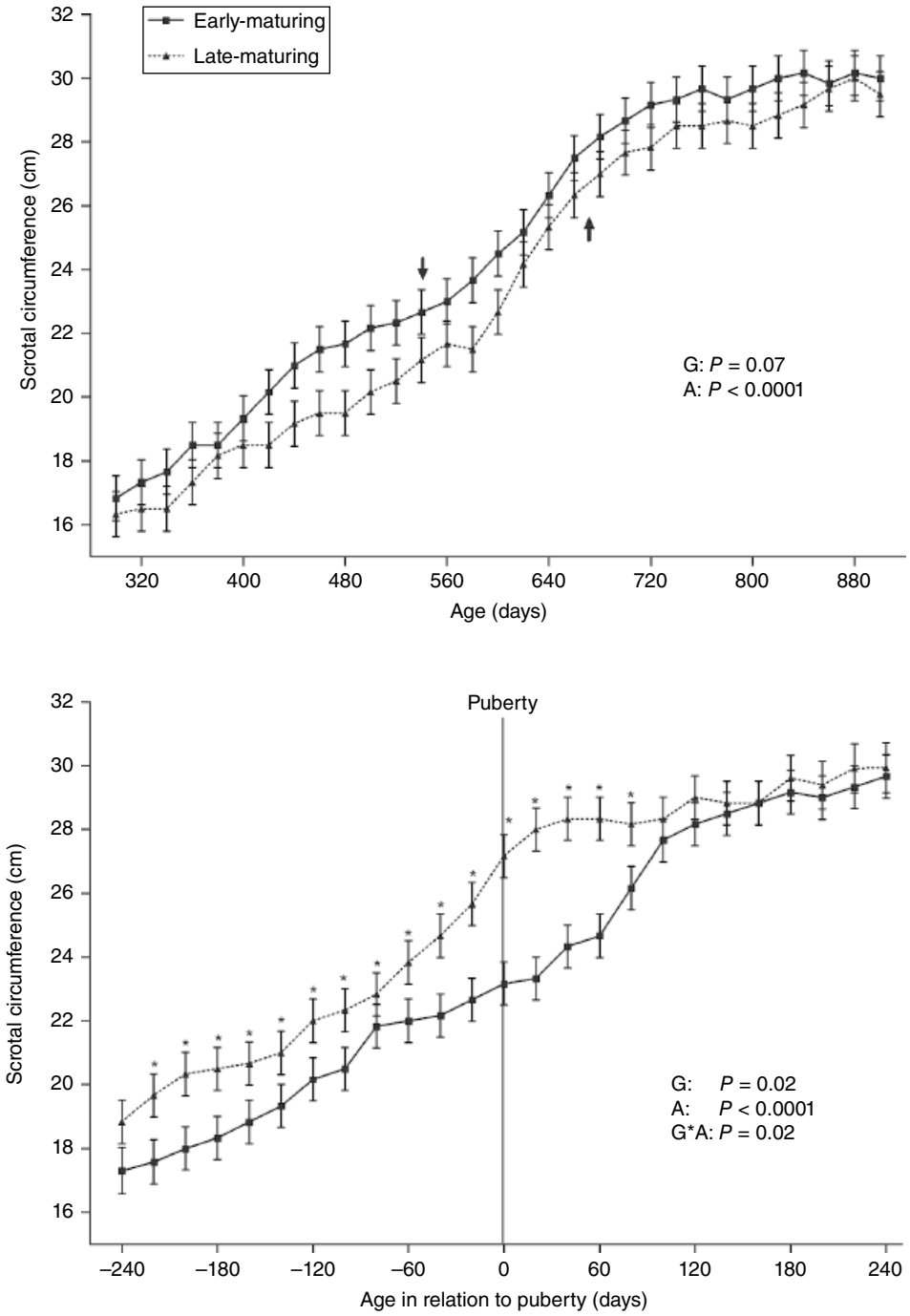


Figure 6.7 Mean (± SEM) scrotal circumference (SC) according to age (*top*) and age at puberty (*bottom*) in early- and late-maturing Nelore bulls (n = 6 per group). Arrows indicate mean age at puberty (ejaculate containing ⩾50 × 10^6 sperm with ⩾10% motile sperm; downward arrow indicates early; upward arrow indicates late). Early-maturing bulls had greater body weight (not shown) and SC than late-maturing bulls during the entire experimental period. In addition, early-maturing bulls were lighter and had smaller SC at puberty than late-maturing bulls, indicating that sexual precocity is not related to attainment of a threshold body or testicular development earlier, but that these thresholds are lower in early-maturing bulls. G, A, and G*A indicate group, age, and group-by-age effects, respectively. Means with superscript asterisks indicate differences between groups within age. *Source:* From [10], © 2004, Elsevier.

population of composite beef cattle, the correlation coefficient among parental breed group means for SC and percentage of pubertal females at 452 days of age was 0.95, whereas the correlation with female age at puberty was −0.91 [5]. A significantly greater proportion of females had reached puberty at 11 and 13 months of age when sired by Limousin bulls with high SC EPD compared with females sired by bulls with low or average EPD [33].

Although sire SC is associated with daughter puberty, evaluation of the genetic correlation between SC and

Table 6.2 Age adjustment factors for scrotal circumference (SC) at 365 days of age according to breed.

Breed	Age adjustment factor
Angus	0.0374
Charolais	0.0505
Gelbvieh	0.0505
Hereford	0.0425
Limousin	0.059
Red Angus	0.0324
Simmental	0.0543

Source: Modified from [123], © 2010, Kansas State University.
365-day SC = actual SC + [(365 – age) × age adjustment factor].

pregnancy rates has produced low estimates that in some cases are not different from zero [29, 31, 41]. A possible explanation for these observations is a non-linear relationship between the traits. One study in Hereford cattle indicated that the effect of SC breeding values on heifer pregnancy exhibits a threshold relationship. As SC increases in value, there is a diminishing return for improved heifer pregnancy, suggesting that selection for a high SC breeding value may not be an advantage for increased heifer pregnancy over selection for a moderate SC breeding value [23]. Although it would seem that the favorable genetic relationship between SC and age at puberty does not completely translate to heifer pregnancy, it is important to note that the experimental design might have confounded some of the referred results, since it is obvious that when the entire group of heifers reach puberty before exposure to breeding, those heifers reaching puberty at younger ages would have no advantage in conception over those reaching puberty at older ages. Moreover, end-of-season pregnancy rates were used in these studies as opposed to per-cycle pregnancy rates and the value of having heifers conceiving early rather than late in the season might have been lost.

Puberty

After spermatogenesis is established, there is a gradual increase in the number of testicular germ cells supported by each Sertoli cell and an increase in the efficiency of the spermatogenesis, i.e. an increase in the number of more advanced germ cells resulting from the division of precursor cells. The yields of different germ cell divisions, low during the onset of spermatogenesis, increases progressively to the adult level [42–44]. Testicular histological changes and increasing efficiency of spermatogenesis are accompanied by increasing testicular echogenicity. Testicular ultrasonogram pixel intensity starts to increase approximately 12–16 weeks before puberty and reaches maximum values right around puberty [45] (Figure 6.8). If the initial changes in testicular echogenicity are associated with Sertoli cell differentiation and meiosis is not completed until formation of a functional blood–testis barrier, then 12–16 weeks seems to be the interval required for the gradual increase in the efficiency of spermatogenesis that eventually leads to the appearance of sperm in the ejaculate. That testicular echogenicity does not change significantly after puberty indicates that a certain developmental stage of the testicular parenchyma must be reached before puberty, a conclusion corroborated by the observation that testicular echotexture at puberty did not differ between early- and late-maturing bulls [10]. In addition, testicular echogenicity did not change with age in mature bulls [46], suggesting that the composition of testicular parenchyma remained relatively consistent after puberty.

In general terms, puberty is defined as the process by which a bull becomes capable of reproducing. This process involves development of the gonads and secondary sexual organs and development of the ability to breed. For research purposes, however, puberty in bulls is usually defined as an event instead of a process. Most researchers define attainment of puberty by the production of an ejaculate containing 50 million or more sperm with 10% or more motile sperm [47]. The interval between the first observation of sperm in the ejaculate and puberty as defined by these criteria is approximately 30–40 days in *B. taurus* bulls [48, 49]. Age at puberty determined experimentally can be affected by the age that semen collection attempts are performed, the interval between attempted collections, the method of semen collection (artificial vagina or electroejaculator), the response of the bull to the specific semen collection method, and the experience of the collector(s). Moreover, age at puberty is affected by management, nutrition (see below), and genetics. Table 6.3 describes weight, SC, and age at puberty in different breeds. Although data from large trials comparing bulls of different breeds raised as contemporary groups are scarce, some liberties could be taken to make some generalizations. Dairy bulls usually mature faster and attain puberty earlier than beef bulls. Bulls from continental beef breeds (with the exception of Charolais) usually attain puberty later than bulls from British beef breeds, especially Angus bulls. Bulls from double-muscled breeds are notorious for being late-maturing. Puberty is delayed in bulls from tropically adapted *B. taurus* breeds and in non-adapted bulls raised in the tropics. In general, *B. indicus* bulls attain puberty at considerably older ages than *B. taurus* bulls.

There is large variation in age and body weight at puberty across breeds and within breeds. Although on

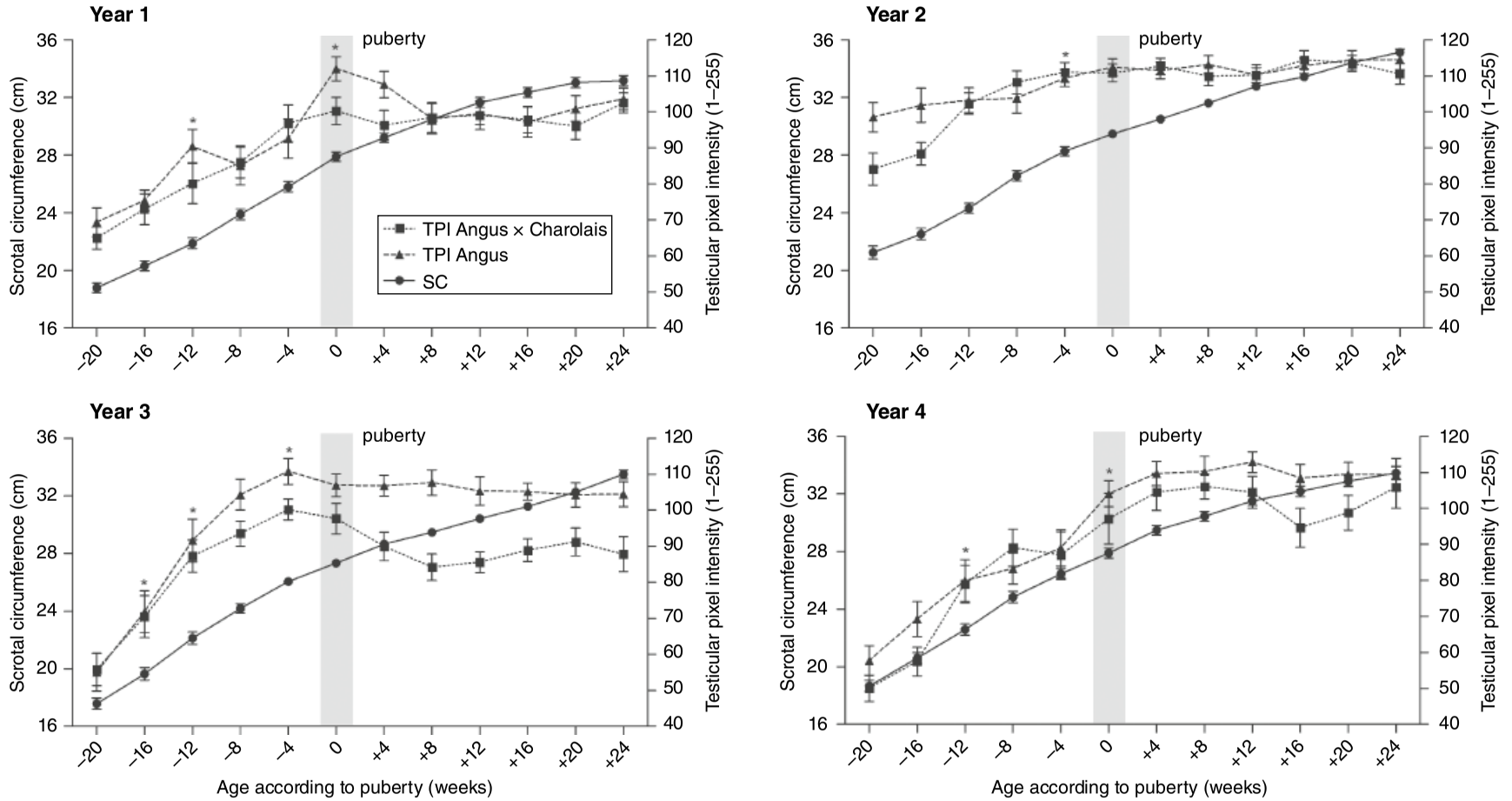


Figure 6.8 Mean (± SEM) scrotal circumference (SC) and testicular ultrasonogram pixel intensity (TPI) according to age at puberty in Angus and Angus × Charolais bulls (Year 1, $n = 37$; Year 2, $n = 39$; Year 3, $n = 43$; Year 4, $n = 33$). TPI, determined on a scale of 1 (black) to 255 (white), started to increase 16–12 weeks before puberty and reached maximum values four weeks before or at puberty. These results indicate that a certain developmental stage of the testicular parenchyma must be reached before puberty and that the composition of the parenchyma remains consistent after puberty. Overall, TPI was greater ($P < 0.0001$) in Angus × Charolais than in Angus bulls. TPI means with superscript asterisks indicate overall change ($P < 0.05$) with age. *Source:* From [45], © 2012, Elsevier.

Table 6.3 Age, weight, and scrotal circumference (SC) at puberty (ejaculate with ⩾50 million sperm and ⩾10% sperm motility) in different breeds.

Breed	Age (months)[a]	Weight (kg)	SC (cm)	References
Angus	10.1	309	30.0	[47]
Bos taurus beef crosses	7.8–9.7	272–339	27.9–28.3	[6, 48, 116]
Brahman	15.9–17.0	350–430	28.2–33.0	[125–128]
Brown Swiss	8.7–10.2	233–295	25.9–27.2	[48, 49]
Charolais	9.4	396	28.8	[74]
Guzera	18.2	310	25.6	[119]
Gyr	17–19.2	315–346	26.2–27.9	[11]
Hereford	9.6–11.7	261–391	27.9–32.0	[9, 47, 48, 98]
Holstein	9.4–10.9	276–303	28.4	[49, 51]
Nelore	14.8–19.7	232–298	21.7–24.3	[10, 119, 129]
Red Poll	9.3	258	27.5	[48]
Romosinuano	14.2	340	28.8	[125]
Simmental	10.6–11.4	328–419	30.6–34.0	[98]

[a] Transformed from days or weeks from original reports.

average *B. taurus* bulls attain puberty with SC between 28 and 30 cm regardless of the breed, the fact that there is still considerable variation in SC at puberty is sometimes overlooked. Interesting observations have been reported in studies evaluating differences between early- and late-maturing bulls. Bulls that attain puberty earlier were generally heavier and had greater SC than bulls that attained puberty later; however, both weight and SC were smaller at puberty in early-maturing bulls [9, 10, 50] (Figure 6.7). These observations not only indicate that precocious bulls develop faster, but also suggest that sexual precocity is not simply related to earlier attainment of a threshold body or testicular development. In fact, these thresholds seem to be lower in early-maturing bulls, and late-maturing bulls must reach a more advanced stage of body and testicular development before puberty is attained.

Spermatogenesis efficiency reaches adult levels at approximately 12 months of age in Holstein bulls [42, 51] and 2.5–3.5 years of age in *B. indicus* bulls [52]. Individual variation in spermatogenesis efficiency is relatively small and is not affected by ejaculation frequency; values between 10 and 14 million sperm per gram of testicular parenchyma have been reported for bulls [1, 6, 42, 51–57]. Since spermatogenesis efficiency is somewhat constant among bulls, daily sperm production of a bull is largely dependent on the weight of the testes. Considering testicular weight at different ages, yearling *B. taurus* bulls are expected to produce around four to five billion sperm per day, whereas adult bulls are expected to produce around seven to nine billion sperm per day. Sperm output (number of sperm in the ejaculate) in bulls ejaculated frequently is essentially the same as sperm production [54]. One important difference between young and older bulls is the capacity of the epididymis to store sperm. Evaluation of sperm numbers in the tail of the epididymis in 15- to 17-month-old Holstein bulls demonstrated that sperm available for ejaculation corresponded to approximately 1.5–2 days of sperm production, whereas in 2- to 12-year-old bulls stored sperm numbers corresponded to approximately 3.5–5 days of sperm production [58, 59]. These observations are especially important for artificial insemination centers and indicate that more frequent semen collection is necessary to maximize sperm harvest from young bulls, whereas semen collection intervals of less than three days have smaller effects on increasing sperm harvest from older bulls. Sperm output increases with increased ejaculation interval up to the number of days required for epididymal storage capacity to reach its limit. Sperm that are not ejaculated are eliminated with urine or during masturbation.

Semen quality in peripubertal bulls is poor and a gradual improvement characterized by increase in sperm motility and reduction in morphological sperm abnormalities is observed after puberty. The most prevalent sperm defects observed in peripubertal bulls are proximal cytoplasmic droplets and abnormal sperm heads (approximately 30–60% and 30–40% at puberty, respectively; Figure 6.9) [9, 50, 60]. The difference between age at puberty and age at satisfactory semen quality (≥30% sperm motility, ≥70% morphologically normal sperm) was 110 days in *B. indicus* bulls [10] and 50 days in *B. taurus* beef bulls; 10% of the

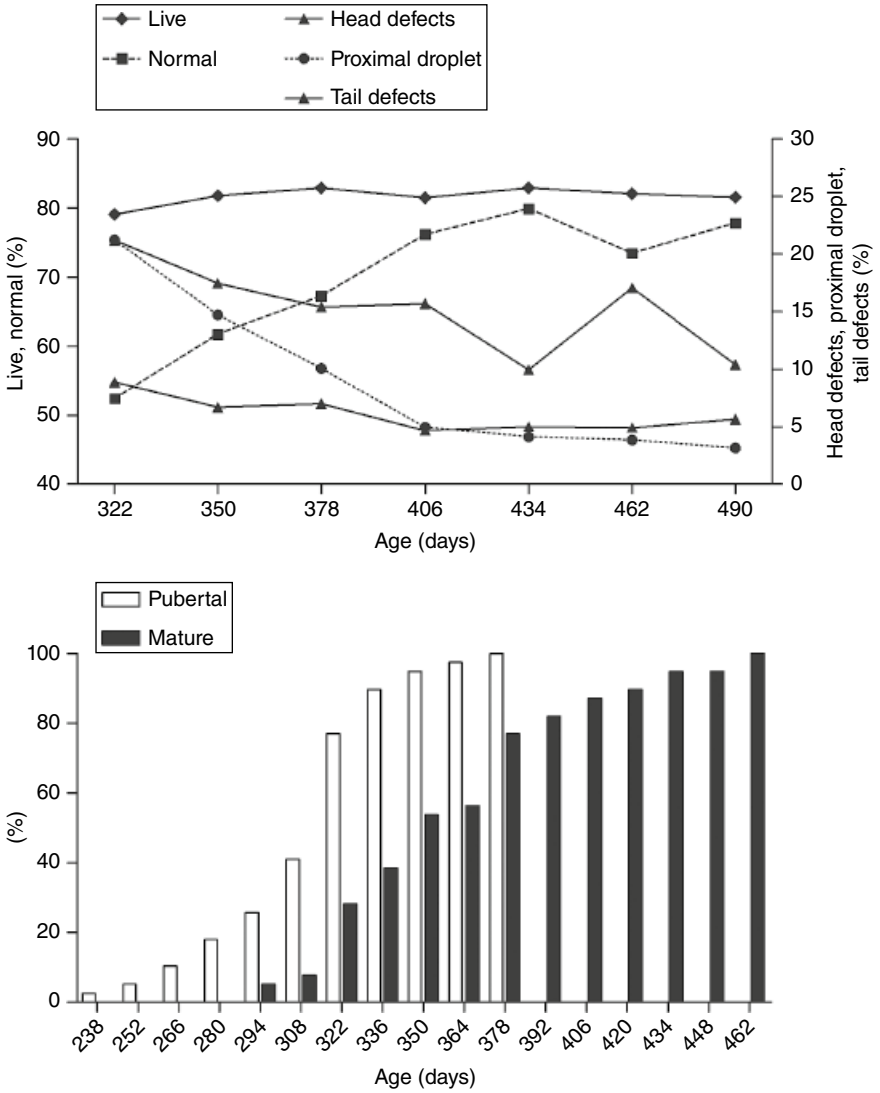


Figure 6.9 *Top*: Mean sperm viability and morphology according to age in Angus and Angus × Charolais bulls (n = 39). *Bottom*: Proportion of pubertal (ejaculate containing ≥50 × 10^6 sperm with ≥10% motile sperm) and mature (ejaculate containing ≥30% motile and ≥70% morphologically normal sperm) bulls according to age. The interval between puberty and maturity was approximately 50 days. *Source:* From [61], © 2012, Elsevier.

latter did not have satisfactory semen quality by 16 months of age [61] (Figure 6.9). In western Canada, the proportions of *B. taurus* beef bulls with satisfactory sperm morphology (≥70% morphologically normal sperm) at 11, 12, 13, and 14 months of age were approximately 40, 50, 60, and 70%, respectively [62]. Similarly, only 48% of *B. taurus* beef bulls 11–13 months old in Sweden had less than 15% proximal cytoplasmic droplets and less than 15% abnormal sperm heads [63]. In Australia, sperm morphology evaluation of bulls of various breeds submitted to breeding soundness revealed a significant negative relationship between age and the occurrence of proximal droplets (Figure 6.10), whereas there was no association between age and other sperm abnormalities [64]. These observations have profound implications on the ability of producers to use yearling bulls and the ability of artificial insemination centers to produce semen for progeny testing at the youngest possible age.

Development of Accessory Sex Organs

Anatomical changes of the testicular vascular cone as the bull ages and starts to produce sperm are indications that the efficiency of the counter-current heat exchange mechanism between the testicular artery and veins in the pampiniform plexus needs to improve in order to cope with

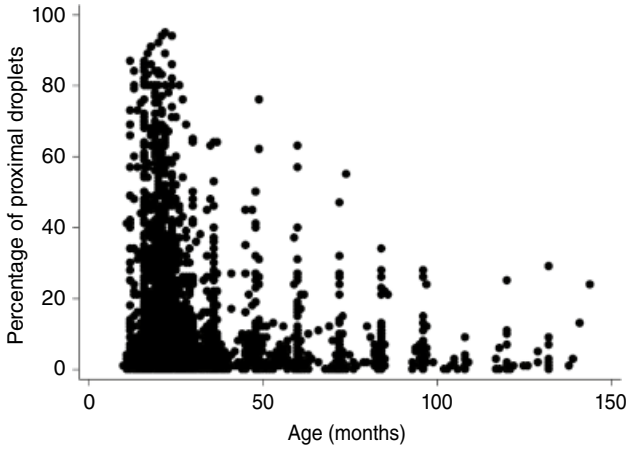


Figure 6.10 Prevalence of proximal droplets in ejaculates from bulls of various breeds according to age (n = 7284). *Source:* From [64], © 2020, Elsevier.

increasing testicular metabolism. In *B. taurus* beef bulls the testicular vascular cone diameter measured by ultrasonography increases until approximately 13.5 months of age, or until 1–8 weeks before SC reaches a plateau [65] (Figure 6.11). In crossbred beef bulls, the length and diameter of the testicular artery in the vascular cone increased from 6 to 12 months of age (1.8 m and 1.9 mm vs 3.1 m and 3.5 mm, respectively), but did not increase significantly thereafter [66]. The testicular artery length and volume in the vascular cone were 1.6 m and 6 ml in 15-month-old Angus bulls and 2.2 m and 11.4 ml in 28-month-old crossbred bulls, respectively [57]. Other studies have reported that the testicular vascular cone length is approximately 10–15 cm and that testicular artery length varies from 1.2 to 4.5 m in adult beef bulls of several breeds [67–69]. In addition to the lengthening of the testicular artery, the distance between the arterial and venous blood in the testicular vascular cone also decreases with age as a result of thinning of the artery wall (317 and 195 µm at 6 and 36 months of age, respectively) and reduction of the distance between the artery and the closest veins [66].

The epididymis continues to grow until at least 6 years of age in Holstein bulls and epididymal weight increases from 9 g at 8 months of age to 15, 23, 27, and 38 g at 12, 18, 25–48, and 73–96 months of age, respectively [51, 70]. The greatest dimension of the epididymis tail measured by ultrasonography from an oblique plane near the distal pole of the testis in Friesian bulls increased from 0.9 cm at 3 months of age to 1.8 and 2.7 cm at 12 and 24 months of age, respectively [71]. The tube-like vesicular glands in newborn calves increase in length and become lobulated during development. The weight of the vesicular glands increased until approximately 4 years of age in Holstein bulls, from 13 g at 8 months of age to 26, 35, 54, and 78 g at 12, 18, 25–48, and 73–96 months of age, respectively [51, 70]. The maximum diameter of vesicular glands in Friesian bulls increased significantly only from age 3 months (1.1 cm) to 9 months (1.6 cm), suggesting that further increase in gland weight is a result of increase in length [71]. Prostate diameter also only increases significantly until 12 months of age (0.85 cm from 0.49 cm at 3 months), whereas maximum diameter of the bulbourethral glands increased from 1.2 cm at 3 months of age to 1.5 and 2.1 cm at 12 and 24 months of age, respectively [71].

In *B. taurus* bulls, the sigmoid flexure of the penis begins to develop at about 3 months of age, penis length increases by up to five times by the onset of puberty, and length continues to increase until sexual maturity [72]. The penis in Friesian bulls 13–19 months old measured 73–89 cm [73], whereas the penis in Holstein bulls aged 25 months or older measured 95–106 cm [70]. First protrusion of the penis during mounting was observed at approximately 8 months of age, whereas complete separation of penis and sheath was observed at approximately 8.5 months of age in Angus, Charolais, and Hereford bulls [47, 74]. Complete sheath–penile detachment evaluated during electroejaculation was observed around the same time of puberty, whereas first completed service evaluated during libido testing was only observed approximately one month after puberty [48].

Development of Sexual Behavior

Development of sexual behavior has been studied in Hereford bulls. Mounting in response to an estrual female was first observed by 3, 6, and 9 months of age in 18.5, 26, and 48% of the bulls, respectively. By 12 months of age 59% of the bulls had their first ejaculations, whereas by 15 months 78% of the bulls registered a complete service. The number of services increased with age until 18 months of age [75]. In Angus, Brown Swiss, Hereford, Angus × Hereford, and Red Poll the first completed service was observed around 11 months of age [48]. Rearing seems to affect development of sexual behavior, since Hereford calves raised in individual pens had a greater number of services when tested for the first time than bulls raised in groups, although the differences quickly disappeared once these bulls were grouped together [76]. In another study, the influence of the presence of females during bull rearing was evaluated. In the first two hours of being exposed to females in estrus, males raised with females had 73% more services than bulls raised in isolation. However, bulls reared in isolation compared favorably in further tests, illustrating the fact that a learning process occurred rather quickly after exposure to females [77].

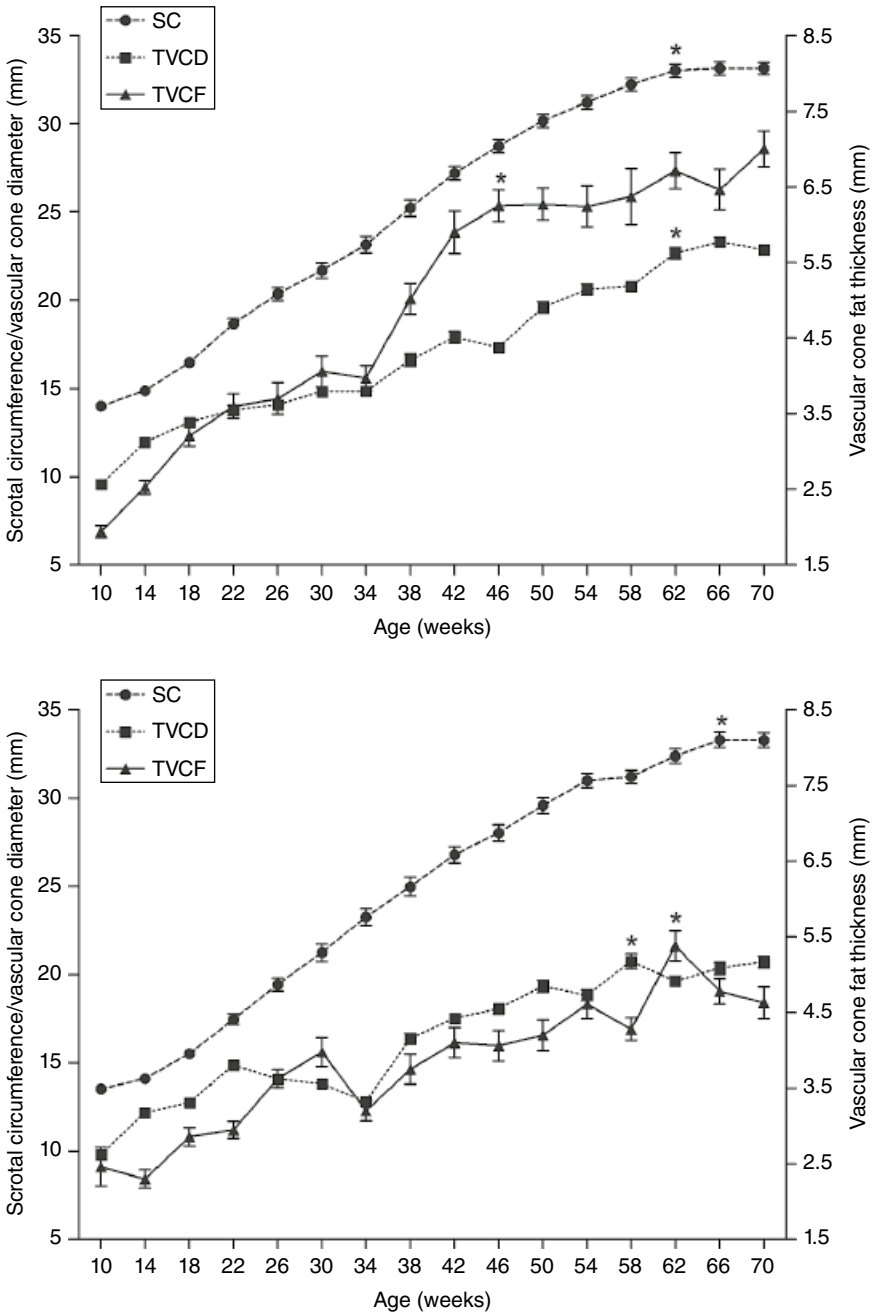


Figure 6.11 Mean (± SEM) scrotal circumference (SC), testicular vascular cone diameter (TVCD), and fat thickness (TVCF) in Angus and Angus × Charolais bulls in two years (*top*: $n = 37$; *bottom*: $n = 33$). Testicular vascular cone diameter increased with age following testicular development, whereas vascular cone fat thickness increased similar to a pattern observed for body backfat. Means with superscript asterisks indicate last significant ($P < 0.05$) change with age. *Source:* From [65], © 2012, Elsevier.

Age and experience have major effects on libido and serving capacity test results. An overall increase in test scores has been observed in several studies with yearling bulls when tests were conducted repeatedly over relatively short intervals, indicating that a maturing and/or learning process occurs rapidly after exposure of bulls to females. Exposure of yearling bulls classified as low serving capacity to estrual females for four days resulted in increased serving capacity score when bulls were tested within a week later; 85% of these bulls moved into the medium- or high-serving capacity category [78]. No correlations between the numbers of services at 12 months of age with sexual performance at older ages were observed in Hereford bulls. Only when bulls reached 18 months did individual

differences in serving capacity remain consistent from one age to another, i.e. 18, 21, and 24 months [79]. In one study, only 53% of 113 yearling bulls completed a service during 10 minutes of libido testing [80], whereas in a different study with yearling Angus and Hereford bulls the variance of the score obtained during eight libido tests conducted over a two-month period ranged between 69 and 73% [81]. These observations suggest that attempts to predict sexual performance are likely to be unproductive until bulls can fully express their inherent sexual behavior and serving ability. When adult Angus bulls were evaluated, the time required to complete six services when exposed individually to 10 estrous females increased with age from 31 minutes in two-year-old bulls to 43, 55, and 67 minutes in three-, four-, and five-year-old bulls, respectively [82]. However, in another study no difference in libido score or servicing capacity test results between two- and three-year-old Angus and Hereford bulls was observed [83].

Effects of Nutrition on Sexual Development

In early studies evaluating the effect of nutrition from birth to maturity on sexual development and reproductive function in bulls, Holstein bulls receiving low nutrition (approximately 60–70% of requirements) were older at the time the first ejaculates containing motile sperm were collected and had smaller testes, whereas bulls receiving high nutrition (approximately 160% of requirements) had earlier puberty and larger testes when compared with bulls receiving control nutrition (100% of requirements) [84, 85]. Several recent studies involving manipulation of nutrition during prepubertal and pubertal periods in both beef and dairy bulls have provided unequivocal evidence that (i) most pronounced effects of nutrition occur during the prepubertal period, (ii) effects of nutrition during the prepubertal period cannot be compensated with changed nutrition during the pubertal period, and (iii) high nutrition during the peripubertal period hastens puberty and results in larger testes (SC and testes weight), whereas low nutrition produced opposite results. With optimal prepubertal nutrition, puberty can be hastened by 1–1.5 months and testicular mass at approximately 1.5 years of age can be increased by 12–18% (Table 6.4) [86–91].

The effects of nutrition on sexual development and reproductive function in bulls are mediated through the hypothalamic–pituitary–testes axis. Nutrition affects the gonadotropin-releasing hormone (GnRH) pulse generator in the hypothalamus, since differences in luteinizing hormone (LH) pulse secretion in bulls receiving different nutrition can be observed even in the absence of differences in pituitary LH secretion capability as determined by GnRH challenge [87, 88]. Interestingly, though, when low nutrition was imposed on bulls by limiting the amount of nutrients in a ration fed ad libitum, only reduced LH pulse frequency was observed, whereas reduced LH pulse frequency, mean and peak concentrations, and secretion after GnRH challenge were observed when nutrition was controlled by restricting the availability of food [86]. These results seem to indicate that the inhibitory effects of limited availability of nutrients on LH secretion appeared to be exerted only on the hypothalamus, whereas the combination of limited availability of nutrients and the sensation of hunger experienced by bulls with restricted intake affected both hypothalamic and pituitary function, producing a much more severe inhibition of LH secretion. The effect of nutrition on Leydig cell number and/or function in bulls receiving different diets was demonstrated by differences in testosterone secretion after GnRH challenge even in the absence of differences in LH secretion after the challenge [87, 88].

Table 6.4 Effect of prepubertal nutrition (before 6 months of age) on age at puberty (transformed from days from original reports) and paired testes weight at 16–19 months of age in bulls. Values reported as ranges indicate bulls received varying nutrition in the pubertal period.

	Age at puberty (months)			Paired testes weight (g)			
Breed	**Low nutrition**	**Control**	**High nutrition**	**Low nutrition**	**Control**	**High nutrition**	**Reference**
Angus and Angus × Charolais	10.3–10.8	9.6	–	528–553	600	–	[86]
Angus and Angus × Charolais	–	10.7	10.3	–	531	611	[87]
Angus and Angus × Charolais	10.5	9.8	9.4	520	549	655	[88]
Holstein	–	10.6	9.9	–	535	636	[89]
Holstein	12.1	10.7	10.6	562	611	727	[90]
Holstein	10.4–10.6	–	9.3–9.8	594–627	–	658–660	[91]

Differences in yearling SC due to age of the dam in beef bulls could also be interpreted as an indication that nutrition during the pre-weaning period affects sexual development, although possible in utero effects cannot be completely ruled out. SC in *B. taurus* beef bulls increases as age of the dam increases until five to nine years of age and decreases as dams get older. Adjustment factors of 0.7–1.4, 0.2–1.0, 0.1–1.0, and 0.3–0.75 cm for yearling SC have been suggested for bulls raised by 2-, 3-, 4-, and ≥10-year-old dams, respectively [19–23, 92]. In these studies, the inclusion of weight as a covariate in the models describing SC resulted in decreased effects of age of the dam, indicating that the effect of age of the dam on testicular growth seems to be primarily the result of age of the dam effects on bull's body weight, likely related to differences in milk production. This theory is also supported by reports that, similarly to that observed in bulls receiving low nutrition, LH secretion after GnRH challenge was greater from 3.5 to 6 months of age in bulls raised by multiparous than in bulls raised by primiparous females [93].

Several studies reported in the literature describe the effects of nutrition only during the pubertal period, in other words after the initial hormonal changes that regulate sexual development have occurred. In general, these studies indicate that low nutrition has adverse effects on growth and sexual development. In one study, bulls receiving one-third of the amount supplied to their twin controls had lower body and vesicular gland weights, vesicular gland fructose and citric acid contents, and circulating and testicular testosterone concentrations, whereas circulating androstenedione concentrations were increased [94]. In another series of experiments, beef bulls 8–12 months old receiving diets with low levels of crude protein (8, 5, and 1.5%) for periods of three to six months had markedly reduced testes, epididymis, and seminal glands weights compared with control bulls fed diets containing 14% crude protein. Moreover, seminiferous tubule diameter and seminiferous epithelium thickness were smaller in bulls with a restricted protein intake [95, 96].

Although low nutrition during the pubertal period has adverse effects on reproductive function, the potential beneficial effects of high nutrition after weaning are questionable at best. Effects of energy on sexual development were not consistent in a study with Simmental and Hereford bulls fed diets with a low, medium, or high energy content (approximately 14, 18, and 23 Mcal/day, respectively) from 7 to 14 months of age. Dietary energy affected sexual development in Simmental bulls, but not in Hereford bulls. Simmental bulls in the high-energy group were heavier and had greater SC and testosterone concentrations than bulls in the low-energy group (in general, the medium-energy group was intermediate). However, increased dietary energy did not hasten age at puberty. The only semen trait affected by dietary energy was semen volume, which was depressed in Simmental bulls in the medium-energy group. Serving capacity was greater for Hereford bulls on the high-energy diet, but medium- and high-energy diets were associated with a decrease in the number of services between two testing periods in Simmental bulls. There was a trend for lower sperm motility and proportion of normal sperm in Simmental bulls fed the low-energy diet [97, 98].

In Holstein bulls producing semen for artificial insemination, high energy intake was associated with visual evidence of weakness of the feet and legs and increased reaction time after three years of age [85]. Under field conditions, post-weaning high-energy diets are frequently associated with impaired reproductive function in bulls, likely related to altered testicular thermoregulation due to excessive fat deposition above and around the testes in the scrotum. In one report, sperm motility decreased and the proportion of sperm defects increased with age in Hereford bulls fed to gain more than 1.75 kg/day, which was significantly different from bulls fed to gain approximately 1 kg/day (control). Even after the high-nutrition diet was changed to a control diet, bulls previously receiving high nutrition continued to have lower semen quality. There was greater deposition of fat around the testicular vascular cone in the scrotal neck in bulls in the high-nutrition group and the difference between body and testes temperature was reduced in this group compared with bulls in the control group. This difference was still present after the high-energy diet was changed and the bulls had lost a considerable amount of weight, indicating that fat accumulated in the scrotum is more difficult to lose than other body fat [99].

In another series of experiments, Angus, Hereford, and Simmental bulls were fed high nutrition (80% grain and 20% forage) or medium nutrition (forage only) from approximately 6.5 until 12–24 months of age. In general, bulls receiving high nutrition had greater body weight and backfat, but paired testes weight was not affected by diet. Moreover, bulls receiving high nutrition had lower daily sperm production and epididymal sperm reserves and a greater proportion of sperm abnormalities. The authors indicated that increased dietary energy may adversely affect sperm production and semen quality due to fat deposition in the scrotum, which reduces the amount of heat that can be radiated from the scrotal skin, thereby increasing the temperature of the testes and scrotum [100–103]. Observations from a different study indicated that bulls fed high-nutrition diets had greater SC and scrotal weight than bulls fed medium-nutrition diets, but paired testes weight was not different between the two groups [104]. Growth rate between 6 and 16 months of age did not affect sexual

development and reproductive function in Angus and Angus × Charolais bulls. However, greater body weight at various ages was associated with reduced age at puberty and maturity and with larger testes at 16 months of age, indicating that improved nutrition might be beneficial, but only when offered before six months of age. Average daily gains of 1–1.6 kg/day did not result in excessive fat accumulation in the scrotum, increased scrotal temperature, or reduction in sperm production and semen quality, and could be considered "safe" targets for growing beef bulls [61].

In summary, low nutrition has adverse effects on sexual development and reproductive function regardless of the bull's age. However, most research indicates that high nutrition is only beneficial during the first six months of life, which presents a challenge to bull producers. Beef bull calves are usually nursing until six to eight months of age and very little attention is paid to their nutrition, whereas nutrition offered to dairy bull calves is often suboptimal. Efforts to obtain maximum weight gain during the first months after birth by offering high-nutrition diets and adopting management practices like creep-feeding will be compensated by reduced age at puberty and greater sperm production capacity in adult bulls. It is also clear that although high nutrition diets after six months of age might be associated with greater SC, this effect is likely the result of fat accumulation in the scrotum and not actually greater testicular size. Moreover, sperm production, semen quality, and serving capacity are all compromised in bulls receiving excessive nutrition after this age. Adjusting diets accordingly to maximize growth but to prevent overconditioning after the peripubertal period is advisable.

Implications of Sexual Development and Puberty for Breeding Soundness Evaluation

In general terms, evaluation of bulls for breeding soundness involves a general physical examination, a specific examination of the reproductive system (including measurement of SC), and examination of the semen. According to the guidelines of the Society for Theriogenology (SFT), bulls must be free of any physical abnormalities, have a minimum SC of 30–34 cm depending on age, have 30% or more progressively motile sperm, and have 70% or more morphologically normal sperm in order to be classified as satisfactory potential breeders [105]. Since developing bulls experience significant changes in SC and semen quality over relatively short periods of time, the effect of age and the interaction of age with breed need to be considered for breeding soundness evaluation. The SFT guidelines list the breeding soundness category "classification deferred," which is used for bulls that the veterinarian considers likely to improve with time after reflecting on the bull's signalment, history, test results, and nature of the deficiencies. Most bulls classified in this category are young animals that may not have reached the suggested minimum for SC and semen quality by the time of the examination.

The proportion of yearling *B. taurus* beef bulls classified as satisfactory potential breeders increases from approximately 55% at 10 months of age, to 72% at 11 months, and 78% at 12 months of age as more bulls reach minimum requirements for SC and semen quality [106, 107]. Since there are breed differences in sexual development, a larger proportion of bulls from early-maturing breeds are expected to obtain satisfactory classification at a younger age than bulls from late-maturing breeds. An extreme example was found with tropically adapted Senepol bulls; the proportion of satisfactory yearling bulls was only 25–50%, but this increased to 70–85% at approximately 24 months of age [108, 109]. It has also been reported that the proportion of satisfactory bulls decreases after five to six years of age as bulls become more prone to develop physical and reproductive problems [110, 111].

Special consideration must also be given to *B. indicus* bulls, since these animals in general attain puberty at considerably older ages than *B. taurus* bulls. Although SC in selected mature *B. indicus* and *B. taurus* bulls does not differ [46], using SC guidelines for young bulls devised for the latter unjustifiably penalizes the former. Satisfactory semen quality is also observed at older ages in *B. indicus* bulls. In Florida, Brahman and Brahman × Nelore bulls only had greater than 70% normal sperm after 24 months of age [108]; these observations are consistent with those in Nelore bulls in Brazil [10]. In addition, the proportion of total sperm defects was approximately 57% in Nelore bulls 12–18 months old [112] and 20% of Nelore bulls were still considered immature at two years of age based on the spermiogram [113]. Less than 5% of *B. indicus* bulls were considered satisfactory potential breeders before 15 months of age and the proportion increased to only 20–50% between 18 and 22 months of age [108]. Accordingly, it might be prudent to delay breeding soundness evaluation for *B. indicus* bulls until two years of age.

In breeds that selection pressure has demonstrably resulted in significant increases in the mean SC, suggestions have been made for veterinarians and breed associations to consider updating minimum SC requirements accordingly. Minimum requirements designed to cull the bottom 5–10% of the population should help maintain adequate selection pressure [35].

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Breeding and Health Management

7

Breeding Soundness Evaluation: Comparative Review of Different Standards

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Introduction

The overarching goal for a bull breeding soundness evaluation (BBSE) is to provide a standardized, unambiguous report on the suitability of a bull to be used in a breeding program. A secondary goal may be to utilize the data to facilitate genetic selection for fertility traits. The evaluation process needs to be repeatable, reproducible, efficient, and safe for both the bull and the clinician. There also needs to be flexibility in the process to accommodate differing managerial requirements. Within each managerial situation there needs to be consistency in the process of data collection, data interpretation, and classification of the evaluation outcomes, with results readily understood by the end user. Such consistency helps to maintain the credibility of the BBSE, assisting veterinarians, cattle producers, and researchers alike.

Foundations for the knowledge that underpins the current practices used in the assessment of bull fertility are attributed to research performed in the first four decades of the twentieth century [1]. Seminal research into specific aspects of bull fertility arose out of the USA [2, 3] and Sweden [4]. Following the understanding that many individual factors affect bull fertility, there was a progressive realization of the need to assimilate this knowledge to enable the development and application of minimum standards for each fertility parameter. The assimilation of fertility parameters into a bull fertility evaluation process formed the foundation of BBSE procedures. The earliest documentation in western literature of standardized procedures, interpretation, and reporting for BBSEs was described in 1954 by a group called the Rocky Mountain Society for the Study of Breeding Soundness of Bulls. Following several iterations, this group evolved in 1974 into the American Society for Theriogenology (SFT), with the basis for current protocols, data recording, and reporting being developed [5].

Following on from these initial developments, BBSEs are now conducted by veterinarians throughout the world to estimate the potential for a bull to sire calves under a range of management conditions. These conditions vary from the assessment of bulls pre-sale for seed-stock producers; commercial producers using natural mating in multi-sire or single-sire situations; assessment for the ability to collect and process frozen semen; and assessment for insurance purposes. These varied contexts coupled with differing production goals mean that the processes for determining and reporting the suitability of a bull for breeding have evolved with a degree of autonomy in different regions. While there are many similarities, there are also variations in the techniques employed, interpretation of the findings, reporting of results, and systems for oversight of the processes within the veterinary profession.

General Concepts

Based on cattle industry requirements for efficiency, financial viability, and reliability, the defined needs for veterinary BBSE include [6]:

1) A system for breeders/vendors to provide assurance that bulls have a high probability of being fertile at the point of sale.
2) A system for buyers/users of bulls to understand evaluation reports and to have confidence that bulls have a high probability of being fertile at the point of sale.
3) A system whereby veterinarians can meet industry needs that is consistent with their own professional opinions, enables them to produce professional reports, and enables electronic reporting and storage of data.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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4) A system that is flexible enough to suit bull transactions, insurance examinations, and routine screening of herd bulls.
5) A system that is applicable to wide variations in geographical, cattle genotype, and management situations throughout the world, and equally applicable to dairy and beef bulls.

The early iteration of the BBSE developed by the SFT set the general template for the procedures, data recording, interpretation, and reporting of results. The details of these procedures are described in Chapters 8 and 9, but in summary the BBSE process can be condensed into the following seven categories:

1) General physical examination
2) Reproductive physical examination
3) Measurement of scrotal circumference (SC)
4) Semen evaluation – bull-side
5) Semen evaluation – morphology and other laboratory evaluations
6) Serving ability or capacity
7) Testing for specific infectious or genetic diseases may be carried out on an as-needed basis.

Of these seven aspects of the BBSE, the first three are considered compulsory in systems investigated in all regions. The remaining four categories may be variably included depending on the managerial situation, or the geographical location. While all BBSE systems agree or imply that full semen evaluation is desirable, some systems allow for semen evaluation to be either truncated to just a bull-side, fresh-semen evaluation or removed from the process in cases where large numbers of bulls in remote areas need to be assessed over short time-periods. Additionally, systems from all regions recognize the logistical effort required for testing serving ability or serving capacity, and so do not routinely include this aspect in the BBSE.

With these variations of procedure in mind, this chapter focuses on comparing how BBSE services for the first five categories are delivered in selected regions throughout the world. There is also reflection on the guidance and oversight different regions provide to veterinarians in order to provide a standardized service.

An International Overview of BBSE

The USA

Within the USA, the SFT has taken leadership in the development of BBSE data collection, recording, interpretation, and reporting. Having been in development for more than 60 years [7], the processes employed by the SFT have also served to set a standard for performing the BBSE worldwide. While there is always concern regarding truncated or poorly executed examinations [8], veterinarians in the USA have long had access to clear guidelines for performing the BBSE procedure. The SFT BBSE process has evolved through three main iterations over the past five decades [8–10]. The most recent progression came in 2018 with the production of an updated manual containing evidence-based information for veterinarians on the BBSE process [10]. One of the stated aims of this document is to instil a belief in cattle producers and veterinarians in the integrity of the veterinary BBSE process. This sentiment seems to be a recurring theme for BBSE practice throughout the world.

Due to the commonality in basic procedures throughout the different regions, a detailed coverage of the SFT BBSE form as described in the SFT BBSE manual is presented [10], as it provides an overview of the type of information recorded in all regions.

The SFT BBSE process offers reporting options in either hard-copy or electronic form. The form can be broken down into sections as shown in Figure 7.1, with the first section consisting of the client's information and general information about the bull including age, breed, and identification. The name or ear tag of the bull along with some form of permanent identification (tattoo, hot brand, freeze brand, electronic identification (EID) tag number, or United States Department of Agriculture (USDA) official metal ear tag) is included. The age of the bull is recorded in months up to two years of age to ensure SC measurements are categorized and interpreted correctly in young bulls.

Below the client and bull information, a box is provided to record any known history about the bull's fertility or medical history. Practitioners utilizing the SFT electronic BBSE software can allow automatic filling of this box with the bull's previous breeding soundness examination if it was completed in the software. At the bottom of the box there is a statement regarding the fact that the responsibility is on the producer to evaluate the libido and mating ability of the bull when placed with females.

The next section of the form is the physical examination portion of the examination and begins with the body condition score. The scale for both beef and dairy bulls is present on both form types, with consideration given to the score as well as circling the appropriate descriptor of obese, good, moderate, or thin. The physical examination is completed with the assessment including examination of the bull's eyes; conformation including feet and legs; examination of the scrotum, testes, and epididymides; SC measurement; transrectal examination of the accessory sex glands and internal inguinal rings; and examination of the penis and

Society for Theriogenology
Veterinarians Dedicated to Animal Reproduction

Bull Breeding Soundness Evaluation

Guidelines Established by Society for Theriogenology
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Jeffrey Brooks
P.O. Box 199
Starkville , MS 39759
(662) 321-4567

BSE Date: 6/10/2013	BSE Case No: 13-401
Bull Name: "TEDDY"	Breed: Angus
Bull I.D. No:	Brand ☐ Tattoo ☐ Ear Tag ■
Bull Birth Date:	Age (Mo.) 84

PHYSICAL EXAMINATION

Body Condition Score: Beef - 6
Thin ☐ Moderate ☐ Good ■ Obese ☐

Pelvic Height	Pelvic Width	Pelvic Area

Feet/Legs	■	RR foot grown out no lameness
Eyes	■	
Vesicular Glands	■	
Ampullae/Prostate	■	
Inguinal Rings	■	
Penis/Prepuce	■	
Testes/Spermatic Cord	■	
Epididymides	■	
Scrotum (Shape)	■	
Other		
SCROTAL CIRCUMFERENCE (CM)	40 . 0	

CLASSIFICATION

Interpretation of data resulting from this examination would indicate that on this date this bull is a:

Satisfactory Potential Breeder

Re-examination recomended on:

This bull has been examined for physical soundness and quality of semen only. Unless otherwise noted, no diagnostic tests were undertaken for libido, mating ability, or infectious disease status of this bull.

Signed: _____
Member - Society for Theriogenology



Mirocephalic Heads | Detached Heads | Coiled Mid Pieces and Tails

. Remarks and interpretation (diagnosis, prognosis, recommendations)

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Figure 7.1 The SFT electronic BBSE form. *Source:* From Society for Theriogenology, Bull Breeding Soundness Evaluation form. © 1992, The Society for Theriogenology.

prepuce during the ejaculation process. Each item has an open box beside it to be checked following examination to indicate that the item was assessed and found to be without abnormalities. If an abnormality is noted, an explanation of the abnormality is placed in the box to the right of the checkbox.

SC is part of the reproductive physical examination and is recorded in centimeters just below the physical examination checkboxes on the form. Tables of the expected SC by age are given on the back of the form. In the SFT system, a bull with an SC below the minimum cannot receive the classification of satisfactory potential breeder even if the rest of the physical examination is passed and semen quality is satisfactory.

When filling in the semen examination portion of the form, the method of semen collection is marked, followed by whether the bull obtained erection, full protrusion of the penis, and ejaculation. Protrusion of the penis and examination of the prepuce is essential before deeming a bull a satisfactory potential breeder in this system.

The SFT manual provides good detail on preparing semen for evaluation so that repeatable results can be achieved. These guidelines describe the common requirement to dilute concentrated samples in order to assist visualization of individual spermatozoa for motility assessment. But there is little detail on the practicalities of the dilution process and dilution rates.

The SFT lower threshold for individual motility is set at 30%. It is notable that the measurement of gross motility is no longer recommended as part of the BBSE semen assessment process in the current SFT guidelines.

The SFT no longer classifies morphology defects as primary/secondary or major/minor, but simply records them as they are seen. They are then grouped into Normal or Abnormal Head, Midpiece, and Tail categories for the report. If there is a high percentage of abnormal sperm, notes on what was found are made in the comments. The SFT lower threshold for normal sperm is 70%.

Following the evaluation, the data are interpreted and the bull is classified into one of three categories – satisfactory potential breeder; classification deferred; or unsatisfactory potential breeder. The classification deferred category is used when potentially treatable abnormalities are detected, or when the practitioner considers that aberrations to the spermiogram may be related to a specific insult and time is needed for recovery.

Australia

The BBSE process within Australia has oversight from the Australian Cattle Veterinarians (ACV), with input from the Australian Reproduction Veterinarians (ARV). From the 1970s to the end of the 1990s, BBSEs in Australia were broadly based on the SFT recommendations. Yet, there was variation throughout the veterinary profession in the ability to reliably collect data, the depth of the evaluation, the interpretation of findings, and the quality of reporting [11]. However, over these three decades, there was a steady progression to a more standardized approach as information was disseminated throughout the veterinary profession via improved undergraduate training and continuing professional education. There was also a push from industry stakeholders to ensure consistency in the process, particularly prior to Breed Society sales of young bulls. This consistency was essential so that bull producers could have confidence their bulls were not disadvantaged at sale-time due to variation between veterinarians in data collection or interpretation. In 2002 there was a major progression toward improving the quality and consistency of BBSEs with the ACV commitment to implement a BBSE accreditation scheme for veterinarians [12].

Prior to the implementation of the BBSE accreditation scheme by the ACV in 2007 came an overhaul of the BBSE processes. Initially, the scientific literature was reviewed [13] and the needs of stakeholders were considered in preparing the substantive manual *Evaluating and Reporting Bull Fertility* [14] that outlined standards for assessing and reporting bull breeding soundness. A further progression was the development of software to assist with the recording, interpretation, and reporting of the process [15].

Driven by legal advice and industry requirements, the ACV elected to develop the BBSE process to provide a description of risk, rather than reporting an overall result for the bull. Therefore the BBSE process was divided into five fertility categories, with results for each individual category reported on the certificate. The results provide information on whether the minimum standards for each category were assessed, or met, but the bull is not given an overall Pass/Fail classification.

The bull fertility categories are Scrotum, Physical, Crush-Side Semen, Sperm Morphology, and Serving and are shown on the certificate in Figure 7.2.

The SC is reported in centimeters. The results for all other categories are reported as either a T (Tick), Q (Qualified), X (Cross), or NT (Not Tested). As with the SFT system, the option to report a category as "Q" is chosen if aberrations deemed to be treatable are encountered. Professional, standardized, easy-to-read reports are produced electronically. There are no booklet recording options, but the veterinarian can print out customized field recording sheets for each job once the client and job details have been entered into the database. There is also the option for electronic data entry if the animal handling facility is suitable.

A compulsory database must be collected for all BBSEs, but, informed by veterinary advice, the client can select

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Veterinarians
Dr Wendy
Dr Jenni
Dr Paul

Report: Bull Breeding Soundness Evaluation

This report was compiled exclusively for the use of the person to whom it is addressed. No other person or corporation has any authority to make use of any or all of this report.
This report is valid only when signed by the evaluating veterinarian and the bull's owner or agent

Summary

To: Paul N Oakhill Assisted Animal Breeding Somewhere Green Qld 4567

Place of examination: Oakhill Date: 03 Jan 2020
Brand: MK under wings Breed: Wagyu

Bull Number/name	Age Yr:Mn	Scrotum	Physical	Crush-side Semen	Sperm Morphology	Serving
Sir Lancelot L21	2:02	35.5	✓	✓	✓	NT

I hereby certify that information included in this report is in full accordance with the standards for evaluation and reporting bull breeding soundness as published by the Australian Cattle Vets

Veterinarian: Wendy
Signature:

I hereby certify that there has been no medical or surgical intervention of congenital abnormalities of the listed bull(s), whether genetic or not, to enable the above-mentioned standards to be met

Owner/Agent: Paul
Signature:

Data recorded

Physical	
Condition score	4
Testes tone	3
Penis	Normal
Feet	Normal
Legs	3
Leg joints	Normal
Gait	Normal
Head	Normal
Semen	
Density	4
Mass activity	3
Motility	80%
Morphology	
Normal sperm	87%

General Comment

Figure 7.2 An example of the BBSE report from the Australian system. The certificate includes the identity of the client and place of examination; bull ID, breed, and age; an area for summary of the veterinary interpretation for each aspect of the examination; a signed statement by the veterinarian that the procedures outlined in the ACV BBSE manual have been followed; and a signed statement by the vendor that there has been no surgical or medical intervention. Multi-bull report formats are also available. *Source:* From AVA, BBSE report from the Australian system, © Australian Veterinary Association Ltd.

additional categories they wish to have included in the evaluation to suit their specific purposes (Figure 7.3). In this context, breed societies can select additional parameters they wish to have evaluated for bulls in their respective stud sales with the knowledge that the outcome of that evaluation will be reflected in the result shown in the respective fertility category.

If owners or veterinarians choose to truncate the evaluation procedure, that is possible. But the certificate clearly shows a potential purchaser the testing procedures that are available and which ones were performed. The bull owner or their agent is required to sign the certificate to affirm that bulls have not undergone procedures to rectify faults that may have otherwise caused them to fail the standards.

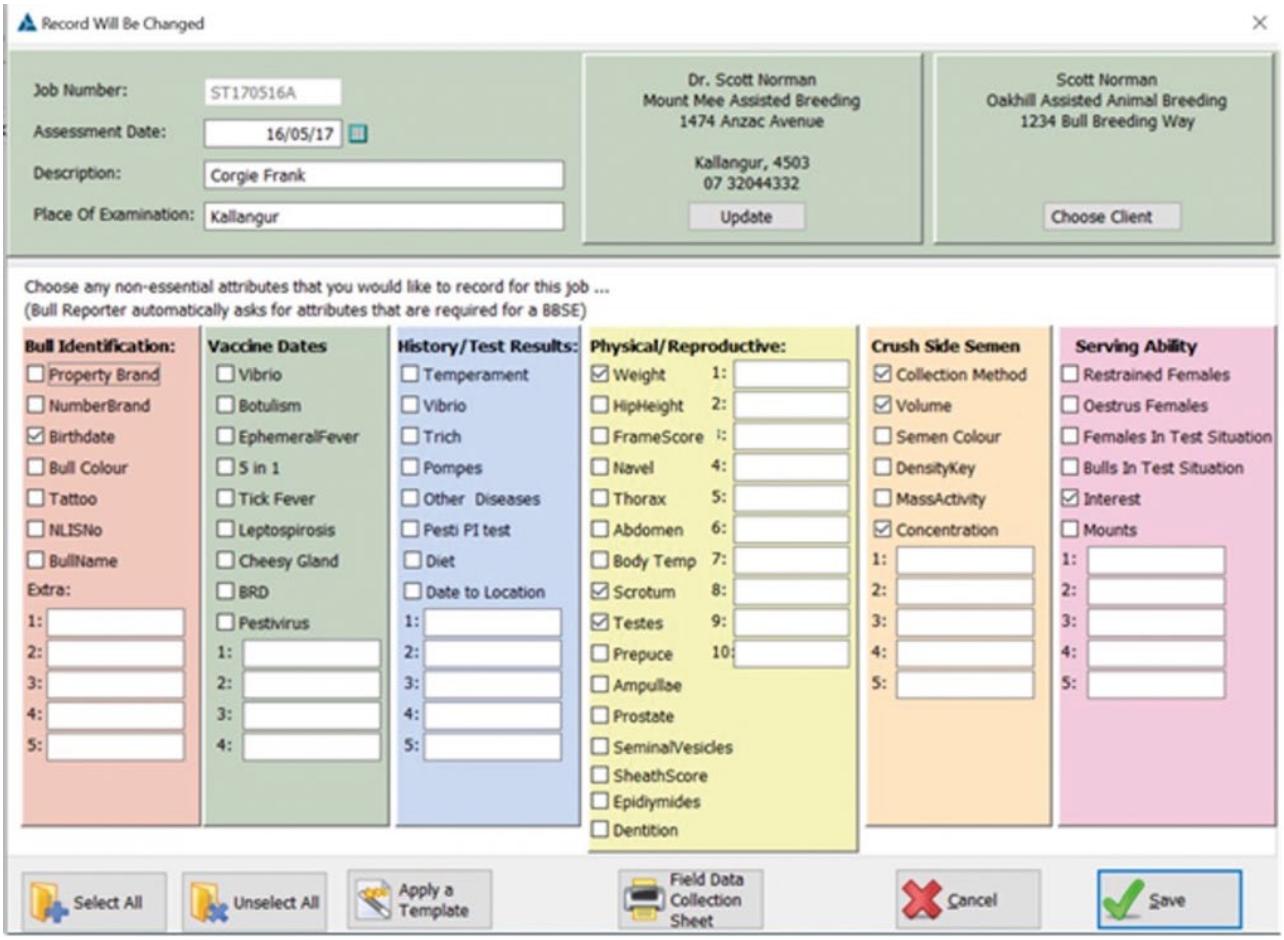


Figure 7.3 An example of BBSE data collection options provided by *BullCheck* software as used by veterinarians accredited in the *BullCheck* accreditation program managed by the ACV. *Source:* Used with permission from ACV, Australia.

The veterinarian is required to sign the form to confirm the evaluation was in accordance with ACV standards.

An important feature of this approach is that the veterinarian is not required to make an overall judgment of whether the bull has normal capacity to sire calves under natural mating management. Instead, the veterinarian assesses each category to determine if standards are met and the client receives a report informing them of whether it meets the individual category standards. This report, with adequate room for veterinary comments, allows clients or potential purchasers to make an informed decision on how to proceed with the bull.

With the current software iteration called *BullCheck*, there is seamless integration with morphology software, which allows the veterinarian to either perform their own morphological assessments or follow the ACV recommendation and export the bull data to a dedicated morphology laboratory that will receive the fixed semen samples and import the results back into the *BullCheck* program for assimilation into the report. To assist with reducing ambiguity in the process, the back page of the report (Figure 7.4) explains in plain language the symbols used on the report, the limitations of the evaluation, and qualifiers to the interpretations.

The Australian system differs to that of other regions with the requirement for veterinarians using this system to become accredited with the ACV. The goal of the accreditation system is to ensure consistency in data collection, interpretation, and reporting. While the process requires effort for both ACV administration and veterinarians wishing to become accredited, the primary goal is anecdotally being achieved. An added benefit is that accredited veterinarians may be securing a market advantage as breeders and breed societies realize the advantages of a well-regulated process. The summary of this system is that veterinarians must demonstrate meeting a threshold of BBSE experience or attend an ACV BBSE training day; they must demonstrate having suitable equipment to perform the procedures, including having a binocular microscope with warm stage, a portable incubator or slide warmer, and

Evaluating and Reporting Veterinary Bull Breeding Soundness

Purpose: **The Veterinary Bull Breeding Soundness Evaluation (VBBSE) is a tool used to identify risk factors that may affect bull fertility in a natural service situation.**

Definitions:

ACV Standards: as published in Beggs et al (2013) *Veterinary Bull Breeding Soundness Evaluation. Published by ACV in 2013 and available from the ACV office.*

Components of the VBBSE

Australian Cattle Veterinarians (ACV) uses the following components to evaluate bull as part of the VBBSE:

Scrotum- Scrotal circumference measured as per published ACV method and reported in cm.
Physical- Examination of bull's structure and genetalia
Semen- Crush-side visual and microscopic assessment
Morphology- Standardised high-magnification microscopy of preserved sperm.
Serving Ability- Standardised test used to evaluate the ability of a bull to mate normally
ACV recommends that all of the above components be assessed.

Report Indicators

Detailed data is reported as either actual measurements, Yes/No, Normal/Suspect/Abnormal, or on a scale as indicated in published ACV standards. A summary risk assessment of each VBBSE component is provided and uses the following key:

✓ Tick - All attributes for this component measured were consistent with the ACV standards. No risk factors for reduced fertility were identified during for this part of the VBBSE examination.	Q Qualified - Not all attributes for this component were consistent with ACV standards but these abnormalities may not necessarily preclude the bulls use. A further comment will be provided. **The client should seek advice from their cattle veterinarian as to the suitability of this bull for a particular purpose. Retesting may be recommended.**
✗ Some attributes for this component measured were not consistent with ACV standards. This bull has a significant risk of reduced fertility in the short term at least. Because some conditions may be temporary, the client should seek advice from their cattle veterinarian.	NT This VBBSE component was not evaluated or not fully evaluated either at the owners request or as indicated.

Using a Veterinary Bull Breeding Soundness Evaluation (VBBSE) report

The report indicates ***the level of risk of reduced fertility*** at the time of testing the bull, and helps predict whether a bull is likely to be able to seek out oestrus females, mate them repeatedly, impregnate them with good quality semen, if the bull were to be put into a paddock mating situation in the short term. The published standards have been selected as the best indicators, but are ***not a guarantee*** that the bull is either fertile, sub-fertile, or infertile.

The ***VBBSE report applies tothe date of the examination*** and no responsibility can be taken for any subsequent events that may affect the ability of the bull to breed satisfactorily or otherwise. In consultation with a cattle veterinarian, clients should consider all aspects of bull fertility when requesting an examination and choose whichever evaluation procedures will best minimise the risk of herd sub-fertility or infertility due to bull failure, or are necessary to meet specific transaction requirements.

The report can only indicate whether bulls meet thresholds for the traits measured based on objective measures and professional judgement of the cattle veterinarian. **Overall judgement of a bull as fertile or sound is not appropriate.** However, bulls may be deemed at risk of being sub-fertile or infertile if the VBBSE detects indicators of this. A prognosis (predicted future status) based on detailed data collected may be provided where thresholds are not achieved or there are other indicators of sub-fertility or infertility.

Limitations of the VBBSE report

The report indicates a level of risk – ***it does not indicate future status***, especially following relocation or management changes. (Bulls, and especially fat bulls, experiencing these events should be re-examined after acclimatisation.) As all possible components of a VBBSE may not be conducted, there always remains a risk that bulls may not reach their full potential.

ACV recommends that all bulls should be vaccinated against/tested for local endemic diseases.

Figure 7.4 As with the South African system, Australian BBSE reports have a back-page description explaining the process, report symbols, and limitations and qualifiers to the process. *Source:* From AVA, The South African system, Australian BBSE reports, © Australian Veterinary Association Ltd.

a computer loaded with a current version of the *BullCheck* software; and they must pass an open-book, online examination. Accredited veterinarians must agree to be in a trace-back system so that disputes can be investigated, and currently in development is a process for accreditation maintenance.

The ACV also temporarily ran an accreditation program for sperm morphologists. This process initiated the development of standardized procedures and quality assurance programs surrounding morphology assessment and reporting. However, the accreditation program had to be abandoned as the ACV did not have effective regulatory authority over non-veterinarians performing the work, meaning they could not follow through with proposed trace-back programs. Currently the ACV provides endorsement for morphologists who have agreed to meet the required laboratory standards.

Canada

In Canada, there have been two systems utilized for BBSE [16]. Some practitioners follow the American SFT guidelines described previously, while the Western Canadian Association of Bovine Practitioners (WCABP) has produced its own guidelines and forms. The WCABP BBSE process is similar to the SFT guidelines except for varied thresholds for SC, morphologically normal sperm, and sperm motility [16].

In contrast to the SFT system, the WCABP system considers breed and age when setting the SC threshold. In the SFT system, the lower SC threshold is the same (30 cm) for bulls of any *Bos taurus* breed between 12 and 15 months of age [10]. However, in the 2018 version of the SFT BBSE manual there is recognition of the profound influence of breed and age on SC, providing veterinarians with information to tailor recommendations to clients with different breeds. The WCABP system sets the SC threshold for each breed and age as one standard deviation below the mean [17], which means that 16% of bulls with the smallest SC may be culled. Both systems classify bulls as having either satisfactory or unsatisfactory SC scores, but do not further categorize satisfactory bulls to allow positive selection pressure based on SC.

For sperm motility and sperm morphology, the SFT system categorizes bulls as either satisfactory or unsatisfactory, while the WCABP system adds another category called questionable for bulls whose values are slightly below the set threshold. As a result, in the WCABP system there are four possible overall classifications for bulls, ranging from satisfactory, questionable, decision deferred, or unsatisfactory breeders. The decision deferred classification is used for bulls that are expected to show a substantial improvement in semen quality before the breeding season, thereby warranting a re-evaluation within several weeks [18].

The UK

The use of BBSE procedures in the UK is a relatively recent development, with the use of formal reporting and certificates commencing in 2009 [19]. Oversight of the standards is provided by the British Cattle Veterinary Association (BCVA) and in general they follow the SFT guidelines, with some influence from the ACV and South African systems. An important distinction with the UK system is that in contrast to other systems that call the process a Breeding Soundness Evaluation, the BCVA considers it more prudent to describe the process as a Pre-Breeding Examination (PBE) [20]. Removal of the word "soundness" presumably reduces legal liabilities within the UK. Like the South African system described later in this chapter, the UK PBE certificates are provided in carbon-copy pads with assessment standards detailed on the reverse of the form. The certificates provide options for selecting one of three reasons for performing the examination, either pre-sale, pre-breeding, or insurance. The main categories assessed are divided into: Physical examination; Semen examination; and Assessment of mating ability. As with other systems, the bull can receive a classification without the mating ability test being performed. Similar to the South African system, the UK PBE certificate requires the bull to be classified as either Suitable or Unsuitable for Breeding. Within the Suitable for Breeding category, the veterinarian identifies whether an assessment of mating ability was performed or whether the classification was based solely on the physical and semen evaluations. While there is no opportunity to place bulls in a "questionable" or "deferred" category, the system encourages the use of the comments section to provide further information and prognoses for bulls not meeting the standards.

The Netherlands

In the Netherlands, BBSE procedures closely follow those described by the SFT [21]. The reason for mentioning the BBSE procedures used in this region is to highlight the emphasis placed on consistently diluting semen in order to gain an accurate individual motility assessment. Bulls can fail a BBSE based on a poor individual motility assessment, and poor semen handling or an inability to clearly visualize individual sperm can adversely affect the result. In the Netherlands, a routine 1 : 80 dilution of semen with physiological saline warmed to 37 °C prior to individual motility assessment is described. In the absence of direct concentration estimates, such a process will result in

diluted concentrations of 2.5×10^6 to 13×10^6 cells/ml in the assessment sample. Concentrations such as this allow clear visualization of individual spermatozoa. This process is relatively easily performed in the field by adding 790 µl of warmed saline or PBS to 10 µl of semen.

South Africa

The Livestock Health and Production Group of the South African Veterinary Association took the initiative to standardize BBSE within South Africa [22]. As was the case in other regions, this group recognized that advances in knowledge in BBSE had led to variation in what services were being offered by veterinarians. Such variation was identified as a source of dissatisfaction to clients and practitioners, with both groups experiencing inconsistency in the costs and outcomes of bull examinations [22]. This group proceeded to produce a paper-based recording and reporting system for BBSE utilizing evidence-based standards for the selected parameters.

In this system, the veterinarian, in consultation with the client, has the option of selecting the purpose of the examination as either:

A) A natural service sire for sale or owner use
B) A donor of semen for sale
C) An examination for insurance purposes.

There are compulsory aspects of the testing, which include:

1) A general clinical examination
2) Physical examination of the genital system
3) Semen collection and evaluation

Other procedures such as disease testing are optional and depend on conversations between the veterinarian, the client, or the insurance company.

The certificate record (Figure 7.5) provides a list of the recommended examinations and tests for the bull. The veterinarian is required to circle on the certificate which testing was performed and to circle the purpose for which it was performed as noted in items A, B, or C above. Certificates are available in book form, with a carbon copy, which remains in the book. The reverse of the form (Figure 7.6) provides the standards for each parameter and explanatory notes, similar to the Australian system.

A unique aspect of the South African system is that certificates are only issued if the bull meets the BBSE standards and is classified as breeding sound. Other than the provision of space for some qualifying comments, there is no facility on the certificate to classify the bull any other way. Therefore bulls are effectively classified as suitable or unsuitable for breeding. Accordingly, the first component of the certificate is a declaration by the veterinarian that the bull complies with the minimum standards set for BBSE examinations for the selected purpose.

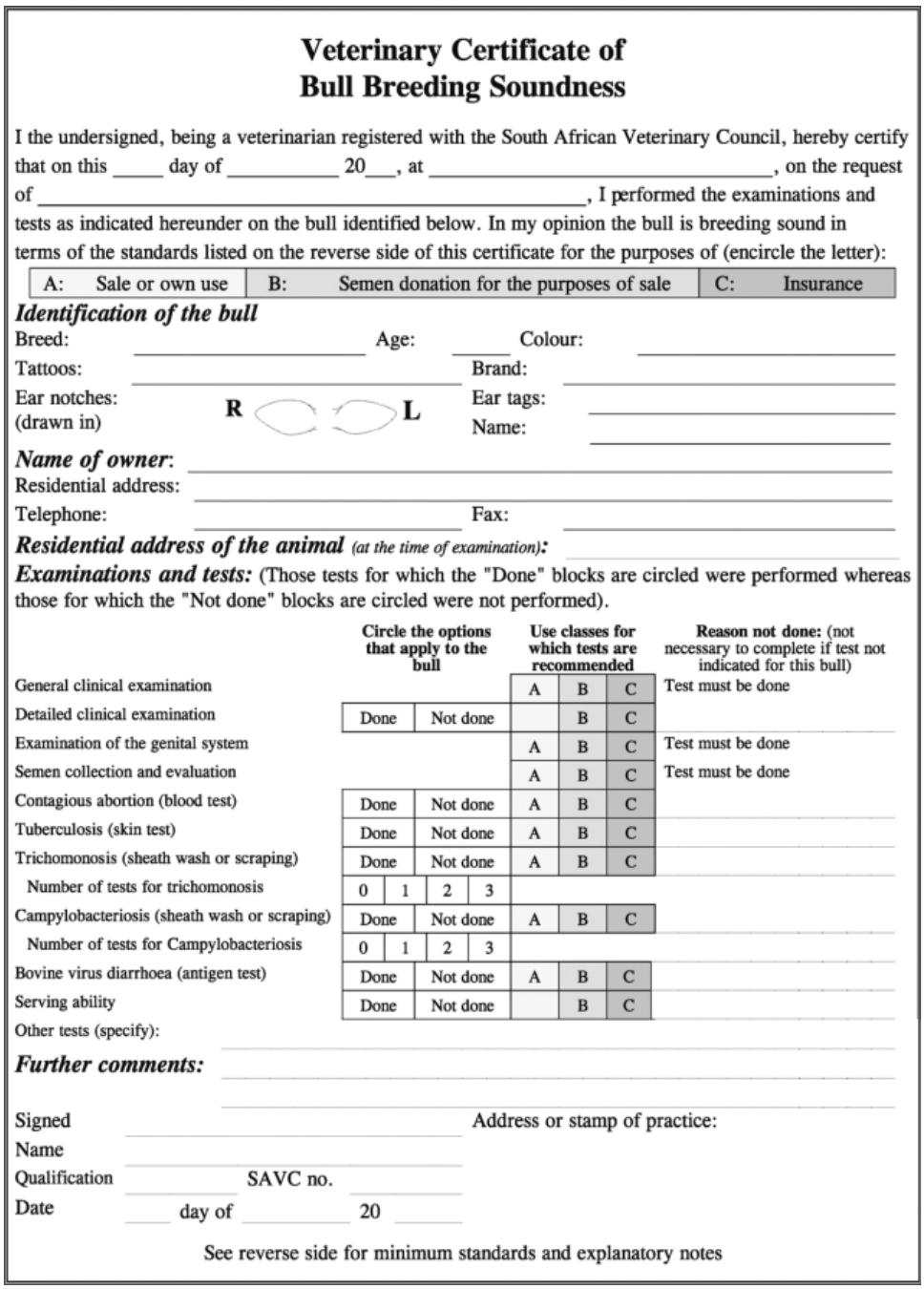
Another unique aspect of this system is that no actual parameters are indicated on the document. The reasoning for this is that the evaluation is designed to provide a yes/no answer to whether the bull is "Breeding Sound" at the time of evaluation. The system acknowledges the risk of artifact affecting the result for many of the parameters when different veterinarians assess bulls in varied field situations. Removing quantitative data from the form is seen as a method of removing inappropriate comparisons between bulls. Therefore if a bull meets or exceeds the parameter standards, the actual number is considered irrelevant. This paradigm contrasts with other systems that aim to both answer the "Breeding Sound" question and provide quantitative data in a way that might assist with genetic selection for improved fertility.

While there is no suggestion of an accreditation process for South African veterinarians to utilize the certificates, there is inference that undergraduate training and routine continuing professional education are adequate to maintain the integrity of the system.

BBSE – How the Categories Compare Internationally

The General Physical Examination

All BBSE standards recommend performing a general physical examination as a compulsory part of the process. There is minor variation regarding the scope of the examination, but in general it consists of an overall visual inspection for abnormalities and conformation, assessment of body condition score, examination of eyes, bite, legs/hooves, and gait assessment. In most systems, there is a general implication that the process be streamlined for efficiency. However, the UK system specifically requires auscultation of the heart and thorax in order to complete the BCVA certificate. If abnormalities are detected, all systems provide for a more detailed physical examination either by using written comments or by selecting further options within the available software. Yet, in all systems, there was either minimal guidance or ambiguity surrounding the decision-making process when conformational and possibly heritable abnormalities were detected. For example, conditions that can have varied degrees of severity such as interdigital fibromas, joint effusion in the hocks, sickle hocks, post-leggedness, and scissor-claw were all mentioned as possible reasons for bulls failing the evaluation. To guide clinicians in these instances, the UK and South



Veterinary Certificate of Bull Breeding Soundness

I the undersigned, being a veterinarian registered with the South African Veterinary Council, hereby certify that on this _____ day of _____ 20___, at _____, on the request of _____, I performed the examinations and tests as indicated hereunder on the bull identified below. In my opinion the bull is breeding sound in terms of the standards listed on the reverse side of this certificate for the purposes of (encircle the letter):

A: Sale or own use	B: Semen donation for the purposes of sale	C: Insurance

Identification of the bull

Breed: _____ Age: _____ Colour: _____

Tattoos: _____ Brand: _____

Ear notches: (drawn in) R L Ear tags: _____

Name: _____

Name of owner: _____

Residential address: _____

Telephone: _____ Fax: _____

Residential address of the animal *(at the time of examination)*: _____

Examinations and tests: (Those tests for which the "Done" blocks are circled were performed whereas those for which the "Not done" blocks are circled were not performed).

	Circle the options that apply to the bull		Use classes for which tests are recommended			Reason not done: (not necessary to complete if test not indicated for this bull)
General clinical examination			A	B	C	Test must be done
Detailed clinical examination	Done	Not done		B	C	
Examination of the genital system			A	B	C	Test must be done
Semen collection and evaluation			A	B	C	Test must be done
Contagious abortion (blood test)	Done	Not done	A	B	C	
Tuberculosis (skin test)	Done	Not done	A	B	C	
Trichomonosis (sheath wash or scraping)	Done	Not done	A	B	C	
Number of tests for trichomonosis	0 1 2 3					
Campylobacteriosis (sheath wash or scraping)	Done	Not done	A	B	C	
Number of tests for Campylobacteriosis	0 1 2 3					
Bovine virus diarrhoea (antigen test)	Done	Not done	A	B	C	
Serving ability	Done	Not done		B	C	

Other tests (specify): _____

Further comments: _____

Signed _____ Address or stamp of practice:

Name _____

Qualification _____ SAVC no. _____

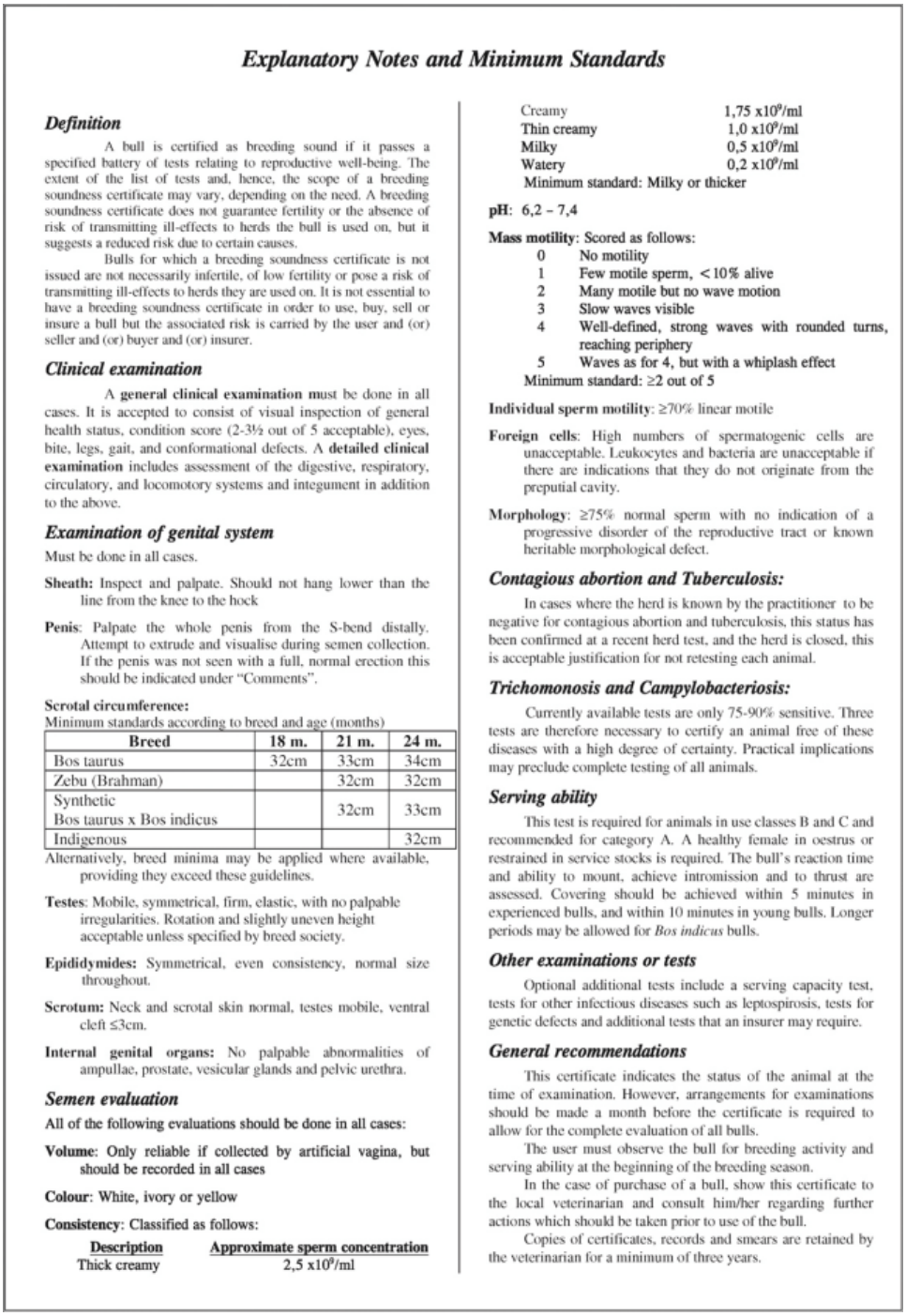
Date ____ day of _____ 20 ____

See reverse side for minimum standards and explanatory notes

Figure 7.5 The standard veterinary BBSE certificate used in South Africa.

African systems provide brief instruction on the back of the certificates, but leave the assessment of severity and final classification to be based on the clinical judgment of the attending veterinarian. Similarly, while the SFT manual provides detailed descriptions of many of the abnormalities that will be encountered, clear guidance on when to pass or fail the bull is lacking. For many of the conditions, the Australian system provides descriptions and, in some instances, scoring templates to assist classification. Yet, commonly the advice simply states that "severe" cases should not pass, leaving it up to the veterinarian to determine what is severe and what is acceptable. This situation may result in enough variation in the results to adversely affect the integrity of the process in the eyes of some producers.

In reviewing the processes of the general physical examination and the possibilities for variation in interpretation of the information both within and between systems, the question is raised of whether it is the role of the BBSE process to fail a bull based on the possibility it may have a



Explanatory Notes and Minimum Standards

Definition

A bull is certified as breeding sound if it passes a specified battery of tests relating to reproductive well-being. The extent of the list of tests and, hence, the scope of a breeding soundness certificate may vary, depending on the need. A breeding soundness certificate does not guarantee fertility or the absence of risk of transmitting ill-effects to herds the bull is used on, but it suggests a reduced risk due to certain causes.

Bulls for which a breeding soundness certificate is not issued are not necessarily infertile, of low fertility or pose a risk of transmitting ill-effects to herds they are used on. It is not essential to have a breeding soundness certificate in order to use, buy, sell or insure a bull but the associated risk is carried by the user and (or) seller and (or) buyer and (or) insurer.

Clinical examination

A **general clinical examination** must be done in all cases. It is accepted to consist of visual inspection of general health status, condition score (2-3½ out of 5 acceptable), eyes, bite, legs, gait, and conformational defects. A **detailed clinical examination** includes assessment of the digestive, respiratory, circulatory, and locomotory systems and integument in addition to the above.

Examination of genital system

Must be done in all cases.

Sheath: Inspect and palpate. Should not hang lower than the line from the knee to the hock

Penis: Palpate the whole penis from the S-bend distally. Attempt to extrude and visualise during semen collection. If the penis was not seen with a full, normal erection this should be indicated under "Comments".

Scrotal circumference:
Minimum standards according to breed and age (months)

Breed	18 m.	21 m.	24 m.
Bos taurus	32cm	33cm	34cm
Zebu (Brahman)		32cm	32cm
Synthetic Bos taurus x Bos indicus		32cm	33cm
Indigenous			32cm

Alternatively, breed minima may be applied where available, providing they exceed these guidelines.

Testes: Mobile, symmetrical, firm, elastic, with no palpable irregularities. Rotation and slightly uneven height acceptable unless specified by breed society.

Epididymides: Symmetrical, even consistency, normal size throughout.

Scrotum: Neck and scrotal skin normal, testes mobile, ventral cleft ≤3cm.

Internal genital organs: No palpable abnormalities of ampullae, prostate, vesicular glands and pelvic urethra.

Semen evaluation

All of the following evaluations should be done in all cases:

Volume: Only reliable if collected by artificial vagina, but should be recorded in all cases

Colour: White, ivory or yellow

Consistency: Classified as follows:

Description	Approximate sperm concentration
Thick creamy	2,5 x10^9/ml
Creamy	1,75 x10^9/ml
Thin creamy	1,0 x10^9/ml
Milky	0,5 x10^9/ml
Watery	0,2 x10^9/ml

Minimum standard: Milky or thicker

pH: 6,2 – 7,4

Mass motility: Scored as follows:

0 No motility
1 Few motile sperm, < 10% alive
2 Many motile but no wave motion
3 Slow waves visible
4 Well-defined, strong waves with rounded turns, reaching periphery
5 Waves as for 4, but with a whiplash effect

Minimum standard: ≥2 out of 5

Individual sperm motility: ≥70% linear motile

Foreign cells: High numbers of spermatogenic cells are unacceptable. Leukocytes and bacteria are unacceptable if there are indications that they do not originate from the preputial cavity.

Morphology: ≥75% normal sperm with no indication of a progressive disorder of the reproductive tract or known heritable morphological defect.

Contagious abortion and Tuberculosis:

In cases where the herd is known by the practitioner to be negative for contagious abortion and tuberculosis, this status has been confirmed at a recent herd test, and the herd is closed, this is acceptable justification for not retesting each animal.

Trichomonosis and Campylobacteriosis:

Currently available tests are only 75-90% sensitive. Three tests are therefore necessary to certify an animal free of these diseases with a high degree of certainty. Practical implications may preclude complete testing of all animals.

Serving ability

This test is required for animals in use classes B and C and recommended for category A. A healthy female in oestrus or restrained in service stocks is required. The bull's reaction time and ability to mount, achieve intromission and to thrust are assessed. Covering should be achieved within 5 minutes in experienced bulls, and within 10 minutes in young bulls. Longer periods may be allowed for *Bos indicus* bulls.

Other examinations or tests

Optional additional tests include a serving capacity test, tests for other infectious diseases such as leptospirosis, tests for genetic defects and additional tests that an insurer may require.

General recommendations

This certificate indicates the status of the animal at the time of examination. However, arrangements for examinations should be made a month before the certificate is required to allow for the complete evaluation of all bulls.

The user must observe the bull for breeding activity and serving ability at the beginning of the breeding season.

In the case of purchase of a bull, show this certificate to the local veterinarian and consult him/her regarding further actions which should be taken prior to use of the bull.

Copies of certificates, records and smears are retained by the veterinarian for a minimum of three years.

Figure 7.6 Explanatory notes accompanying the South African BBSE certificate.

defect that may affect it at some stage in the future. It may be prudent to cull such a bull if it is to sire seed-stock, but not so relevant for a bull entering a commercial herd. Consequently, there is a need for all parties to be clear on why the evaluation is being performed, and also to have the option to vary the examination so it is relevant to the managerial context.

The Reproductive Physical Examination

All BBSE standards recommend performing a physical reproductive examination. There is general agreement across the regions that it should involve the following:

1) Transrectal palpation of the accessory sex glands, with emphasis on assessing the vesicular glands.

2) Examination of the sheath, with some standards providing the non-compulsory option of assigning a sheath score.
3) Palpation or ideally visualization of the penis.
4) Palpation of the scrotum and assessment of the scrotal cleft.
5) Palpation of the testes, epididymides, and scrotal cords.
6) Measurement of SC.

Transrectal palpation of the accessory sex glands is a compulsory part of the BBSE in all regions evaluated. While all structures are evaluated, the clear emphasis is on assessing the vesicular glands, as they most commonly have pathology and their condition is considered to reflect pathology that may be present in other parts of the tubular tract. None of the standards provided clear guidelines on what level of pathology of the seminal vesicles would result in the requirement for reassessment or a fail. The general implication from all regions was that the presence of any of the following would mean the bull would not meet the requirements for the reproductive physical examination: palpable, acute infection; a high concentration of neutrophils in the semen; or enlarged seminal vesicles in conjunction with sperm morphology defects consistent with inflammatory changes to the tubular tract. One area that was not clear in some of the guidelines was what was meant by a "high concentration of neutrophils."

Evaluation of the sheath is recognized as a compulsory part of a BBSE and bull selection in all regions except the BCVA [10, 22–26]. In most regions, failure of the bull in this aspect is based on the veterinarian's opinion of the presence of pathology. In the ACV and SFT systems, a non-compulsory scoring system is utilized to assist veterinarians with pass/fail and genetic selection decisions, but the provision of a sheath score is not considered an essential aspect of the BBSE in any region. The ACV sheath-scoring system has developed over the past 20 years, with current recommendations providing a score on a scale of one to five. Where a score of one represents a small sheath and five represents a large pendulous sheath [23]. There is a plan to upgrade the ACV scoring system [27], but there is still conjecture regarding the relevance of including factors in the scoring system such as the presence of preputial eversion.

There was no variation in the regional recommendations for the assessment and interpretation of penile defects. All standards recommended visualization of the penis during the assessment. Visualization was ideally at electroejaculation or natural service but could at a minimum be during rectal massage. The South African and ACV systems provide the option of providing a BBSE report with accompanying comments in situations where the penis can only be palpated through the sheath. But the SFT system does not allow a classification of the bull as Satisfactory unless the penis has been visualized.

Scrotal Circumference Measurement

All systems agree that the measurement of SC is a compulsory requirement of the BBSE. Additionally, the systems that provide manuals or accreditation programs (ACV and the SFT) emphasize the importance of correct technique when performing the procedure and provide detailed information on how to obtain reliable measurements. They also highlight the need for adequate practical training to master the skill necessary to obtain repeatable measurements.

The interpretation of the SC measurement varies between regions. The traditional interpretation is based on SFT guidelines, where SC standards are based on bull age, with minimal consideration for breed, body condition, or weight. This is the case for the SFT, South Africa, BCVA, and Canadians utilizing the SFT system. The ACV and WCABP systems introduce more complexity in the interpretation, considering more breed and age categories. An example of how the WCABP system differs from the SFT system is that later maturing breeds such as Limousin, Salers, and Blonde d'Aquitaine have a lower SC threshold of 32 cm at 24 months of age compared to the 34-cm threshold in the SFT, South African, and BCVA systems. The ACV system also considers body weight at the time of SC measurement, recognizing the effect body weight and body condition have on SC [23]. On this point, the ACV system was unique in that for young sale bulls it emphasized the need to interpret SC measurements considering the relationships between breed, age, and weight. In the ACV manual, there is a detailed table providing this information for five breed-groups of bulls ranging in age from 12 to 27 months. Consistent with the ACV system moving away from a Pass/Fail approach and more toward a risk assessment paradigm, the SC is simply noted on the top of the report (Figure 7.2), with the veterinary interpretation included in the comments section of the report. The information can still be used by breeders to apply selection pressure, but a low reading does not necessarily result in a fail in that category and the bull may still be suitable for sale for use in a commercial herd if semen assessment and other evaluations meet the standards.

Perhaps the most influence these variations in SC interpretation have on the outcome of the BBSE can be attributed to whether the information is used for a direct assessment of whether the bull is ready to join the breeding herd on a given date or whether the information is being used to leverage fertility selection pressure. In this context, a direct measurement regardless of age, weight, or breed

would provide useful information on whether a bull is mature and suitable to be introduced into the breeding herd on a given date. In contrast, if information is to be used to generate accurate selection data, variably referred to as estimated breeding valuations (EBVs) or expected progeny differences (EPDs), then interpretation based on weight, age, and breed become important.

These variations on how the SC information can be used leads to the need to be clear on the purpose for which the BBSE is being performed. The argument could be made that the primary purpose of the BBSE is to determine if an individual bull is suitable for purpose on a specific date. In this instance, a one-off SC measurement close to the time of entering the breeding herd may be suitable. Conversely, the question is raised of whether it is the role of the veterinarian to fail a bull that is producing semen of acceptable quality if the SC measurement, often taken without important context such as accurate age, breed, weight, and body condition score information, doesn't meet the threshold.

Bull-Side Semen Evaluation

All standards recommend the collection of semen as part of the routine BBSE. However, not all standards explicitly state semen evaluation as a compulsory element of the examination. The ACV standards provide for the option of no semen evaluation, but mandate the compulsory reporting on their certificates of whether semen assessment is included in the evaluation.

For most regions, there was the option for assessment of gross motility (mass activity), apparently based on tradition. But in regions where standards were accompanied by a manual, practitioners were cautioned against using this parameter as part of the BBSE. Notably, the SFT no longer recommends the use of gross motility as part of the semen assessment process and has now removed it from the BBSE form.

All regions recommend assessing individual motility as a compulsory aspect of the BBSE. In regions where manuals are available there is clear recognition that individual motility is readily affected by artifact. To counteract this, detailed procedural guidelines are provided including the requirement for clean, warmed contact surfaces, the use of a binocular microscope, and the recommendation to dilute the semen to ensure the activity of individual cells can be readily assessed. Two of the four regions recommending semen dilution prior to motility assessment provide explicit guidelines for this dilution process, where semen is recommended to be diluted to turbid consistency (between 60 and 200 million/ml) with warmed phosphate buffered saline to ensure repeatable estimates [16, 23]. In the Netherlands, the recommendation is for a 1 : 80 dilution of semen to diluent, providing sperm concentrations of less than 20×10^6 for assessment.

Variation exists in the interpretation of the individual motility assessment. The South African system has the toughest standard, requiring individual motility to be greater than 70%. The ACV, BCVA, and WCABP systems don't consider that the bull meets the requirements for this standard unless it is greater than 60% motile. But the ACV system also accepts that although semen quality may not be suitable for freezing, bulls used solely for natural mating can have commercially acceptable fertility if the motility is greater than 30%. The ACV system therefore allows a "qualified" rating for this standard for individual motility scores between 30 and 60%. The SFT lower threshold for individual motility has been 30% since the first iteration of its BBSE system in the 1970s. This figure recognizes the need to confirm there are motile spermatozoa, but also recognizes that assessments are made in varied field conditions that can adversely affect motility.

Laboratory Assessment of Semen

Semen Morphology

The standards from all regions recommend collection of semen and the assessment of sperm morphology when performing a BBSE. The South African, BCVA, and SFT standards explicitly state that bulls must exceed the morphology threshold of 70% normal spermatozoa to pass the examination and receive the respective certification. The ACV risk-assessment model explicitly provides for the option of no morphological evaluation but mandates the compulsory reporting on their certificates of whether morphology is included in the assessment. In this latter standard, options are provided for separating the semen evaluation into "bull-side" assessment (see section "General Concepts") and morphological assessment. If morphological assessment is performed in the ACV system, a count of 70% normal is required to meet the category requirements, but a "Qualified" assessment can be assigned to this category for bulls with normal sperm percentages between 50 and 69%, which is justified based on industry-specific research [25].

All regions other than the ACV recommended morphology be assessed with either a nigrosin-eosin stained slide or with a preserved, fixed sample under phase-contrast or differential interference contrast (DIC) microscopy. The ACV does not provide the option of nigrosin-eosin stained slides for morphology, instead recommending that bull-side samples be preserved in warmed fixative, such as formal buffered saline, or phosphate-buffered saline (PBS) glutaraldehyde for subsequent assessment.

There are definitive recommendations for the morphological classification of sperm in the SFT, ACV, and BCVA systems. In these systems sperm abnormalities are recorded as seen. There is no clear recommendation regarding morphology classification in the South African system, but the inference is a focus on assessing the number of normal spermatozoa, with abnormalities individually recorded. The conclusion is that morphology assessment for all regions has moved away from previous classification systems such as primary/secondary or major/minor abnormalities. There is still reference to the compensable/uncompensable classification method in the SFT and ACV manuals, with suggestion it may be suited to assessing sperm morphology in samples that will be inseminated, but not relevant to the BBSE process [10, 23].

One specific morphological defect that is the subject of varied opinion is that of distal cytoplasmic droplets (DDs). The SFT no longer considers DDs as having an adverse effect on fertility and counts them as normal (technically a variation of normal) in the spermiogram. The ACV had previously recommended counting DDs as normal sperm, but currently they are recorded as abnormal sperm. The ACV recommends that if the percentage of DDs is great enough to influence whether a bull meets the morphology standard, then the percentage of DDs, accompanied by an interpretation, should be clearly noted in the comments section for this category so that vendors and purchasers can make an informed decision.

The range of regulatory control over morphology assessment processes and efforts to standardize the procedure varies considerably between regions. The South African and BCVA systems simply state that morphology be performed as part of the evaluation, relying on veterinary undergraduate training and personal continuing professional development (CPD) to maintain integrity of the process and interpretation. The SFT provides a detailed manual on sperm morphology assessment and details current knowledge of the influence of morphology on bull fertility to assist practitioners with their interpretations. The ACV system also provides a manual with detailed morphological assessment and interpretative information. But uniquely, while any veterinarian may perform their own morphology assessments, the ACV strongly recommends samples be sent to an ACV endorsed andrology/morphology laboratory to ensure all bulls are assessed by a standard procedure under high-quality optics. The inference from the ACV recommendation is that current Australian undergraduate veterinary training and the quality of microscope optics in the clinical setting may not adequately equip veterinarians for performing repeatable and reproducible morphological evaluations that will stand up to commercial scrutiny. While this position is not necessarily relatable to other regions, it does raise the question of whether the assessment, recording, and reporting of sperm morphology should be subjected to quality assurance practices that are followed for many other laboratory processes. The commercial implication of aberrations in morphology assessment or interpretation is that the sale price of any bull failing to meet minimum standards for sperm morphology will be reduced or the real-world fertility may not reconcile with the anticipated fertility. It therefore seems reasonable for a producer to expect that standards are being applied in an equitable way regardless of the personnel performing the evaluation.

The Use of Vital Stains

In all systems except that of the ACV, the use of nigrosin-eosin staining is one of the recommendations for preparing samples for morphological examination. None of the regions recommends incorporating the Live/Dead aspect of vital staining into the BBSE assessment. The ACV does not recommend the use of vital stains for morphology assessment due to the recommendation of utilizing an endorsed morphologist for the assessment process. In this scenario, the morphologists unanimously prefer samples in fixative such as formal buffered saline (FBS) solution as this enhances the detection of defects under phase-contrast or DIC microscopy. There is also concern that the osmolality of the vital stain may vary widely if not stored carefully, adversely affecting sperm morphology.

White Blood Cells in Semen

The ACV and SFT manuals both describe the detrimental effects of white blood cells (WBCs) in the semen. The ACV suggests that if more than several WBCs are identified per high-power field or if purulent material is observed grossly in the ejaculate, the veterinarian should use clinical judgment regarding treatment and prognosis, with a Q or X applied to the category.

Discussion

While exploring the evolution of the BBSE throughout the world, it is apparent from all regions that variation in the early processes of assessment, interpretation, and reporting of results led to uncertainty in the outcomes of the testing. There was a perception from seed-stock producers and their veterinarians that bull sale opportunities may not be equitable if some bulls were placed under more or varied scrutiny compared to contemporaries. In most regions this variation was arguably the biggest driver toward a standardized BBSE procedure. Regardless of other reasons for performing a BBSE, the requirement for a process that

provides equitable comparison of all sale bulls is critical to the integrity of the BBSE. The understanding is that the primary driver to standardizing the approach to BBSE may not necessarily be the science behind the ideal of improving bull fertility, but more the necessity to make the collection and interpretation of data on which bulls are compared when presented for sale as equitable as possible. If this goal is not achieved, then the credibility of BBSE will be reduced to the point of it no longer being considered by producer bodies to be relevant.

When reviewing BBSE standards from different regions, most provide clear guidelines on what aspects are compulsory and what aspects are suggested recommendations. Ideally these compulsory and suggested processes can be tailored to different bull management scenarios. The South African and BCVA systems have addressed this requirement by stipulating "Use Classes" (Figure 7.5). On similar lines, the ACV system works on a risk assessment format where non-compulsory aspects of the testing can be included or removed depending on the management scenario or breed society requirement. While the SFT system doesn't explicitly provide the option for tailoring the evaluation, there is room to provide comments on options for bulls that fall into the decision-deferred category.

What is not so clear from the standards of different regions is the process of interpreting some aspects of the findings. This is not surprising, as cattle veterinarians are in the unenviable position of having to apply clinical judgment to the continuum of a biological system, while cattle producers are expecting clear "yes/no" answers. To assist with standardized interpretation and reporting it seems there are two requirements: (i) a standardized data recording and reporting system, which all regions provide; and (ii) CPD to ensure there are standardized data collection techniques and interpretation. While two of the regions have provided comprehensive manuals to assist with CPD, other regions appear more dependent on undergraduate training providing a sound knowledge base. Yet, with the knowledge surrounding BBSE constantly evolving, there is a need for experience and on-going training to attain and retain competency. For example, anecdote from the ACV suggests there can be 2- to 5-cm differences in the SC measurements between experienced and inexperienced operators. Similarly, there can be confusion and variable recommendations when conditions such as hocks with joint effusion, interdigital fibromas, aberrations in leg conformation, or neutrophils in the semen are encountered. Assessing extremes in these conditions is not difficult, but standardizing the interpretation of mild to moderate presentations appears less straight-forward. One way to reduce this variation is to provide supervised training, recurrent updates, and where relevant, abnormality scales with exemplars.

For the BBSE to maintain integrity, it is essential that producers have a clear understanding of what to expect when they have their bull assessed. The corollary to having a clear process to follow is that veterinarians will need ready access to CPD in order to maintain currency in the process. The SFT in the USA has taken the lead in the CPD area for close to 50 years, as evidenced in their current and prior manuals for the breeding soundness evaluation of bulls [10]. But perhaps the most extreme evolution of CPD identified in this review is the BBSE Accreditation scheme developed by the ACV in Australia. This scheme provides veterinarians with the opportunity to submit themselves for compulsory postgraduate education and assessment in order to receive accreditation. The accreditation provides them with the commercial advantage of marketing themselves as having industry-recognized skills and competency in the BBSE process.

For credibility of the BBSE process to be maintained, the following points seem important:

1) The system must have standardized data collection processes.
2) There must be standardized data interpretation.
3) There must be standardized reports to allow the end users to easily interpret the findings in order to compare bulls.
4) It seems suitable to move away from a system that places veterinarians in the position of having to "pass" or "fail" bulls using a system that is based on incomplete science – particularly when there is limited opportunity for a detailed assessment and there is incomplete understanding of all factors affecting future fertility. To this end, using a reporting system that simply states the findings for each category and allows for interpretive comments may be a good option.
5) There must be continuing professional education in the processes of BBSE for veterinarians so that producers have confidence in the outcome for their bull.
6) Consideration should be given to an accreditation program for veterinarians performing these procedures. This point is particularly important considering the profound influence outcomes from these evaluations can have on the financial viability of seed-stock producers.

Considering points 5 and 6, the three parameters that commonly concern bull producers are the measurement of SC, the individual motility percentage, and the assessment of sperm morphology. Effective, worldwide extension surrounding the influence of these three parameters on bull fertility means that poor results can lead to a bull not passing the BBSE and either not being able to be sold through normal channels or having a reduced sale price. Alternatively, the bull may have a reduced sale price even if the result meets fertility standards but is lower than contemporaries.

Due to the physiologic and economic importance of these parameters and their highly visible nature to producers, it is essential that veterinarians are adequately trained in these techniques to provide consistent results.

A summary of notable variations in the bull evaluation processes from different regions that may help inform future iterations include:

- Removal of the word "soundness" from the description of the process.
- Provision of opportunities to tailor the evaluation process based on varied managerial contexts.
- Consideration to moving away from a "Pass/Fail" concept to a risk assessment paradigm.
- The provision of more robust guidelines and abnormality scales for interpreting some of the findings.
- Consideration of increasing the opportunities for veterinarians to gain and maintain competence in the processes, which may or may not include an accreditation program.
- Consideration of a quality assurance program for morphology assessment that may, in part, include all veterinarians and morphologists registered with respective regulatory bodies receiving QA samples for evaluation on a regular basis.

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8

Evaluation of Breeding Soundness: The Physical Examination

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Introduction

When a bull breeding soundness examination (BBSE) is performed for the purpose of evaluating a bull's potential to breed in a natural service situation, the physical component takes on paramount significance. In fact, as soon as any physical abnormality that would impede natural service is observed, or the bull does not meet the minimum standards for scrotal circumference (SC), the examination need not proceed to evaluation of semen characteristics. Thus, goals of the physical examination portion of a BBSE are the identification of undesirable genetic traits, structural or physical impediments to breeding, and pathology of the reproductive tract. Even though it is common for inadequacies to exist in this area of the BBSE, it is often ignored or rushed through without attention to detail or in a consistent systematic manner. Therefore it is of utmost importance that the physical examination be done using a consistent protocol. Additionally, the SC, which is an indirect metric for testicle size and thus sperm production potential, must be measured and meet minimum standards.

History

The history can be addressed prior to or during the physical examination. The history can provide the examiner with insight into genetics, condition, management, vaccination, any disease testing programs, and history of disease in the herd. The breed or breed composition should be obtained to help establish what genetic abnormalities or which conformation traits, maturity rates, and age of puberty may be common to that breed. The ration fed previously and at present should be established as well as the level of each ingredient in the rations and the poundage fed to each bull. The veterinarian needs to determine what body condition scores were attained at different stages of development. Management practices should be established with regard to large or small group facilities, parasite control, pasture or dry lot, through space, and water availability. These management practices can influence the level of foot problems and increased incidence of diseases such as vesiculitis. The herd vaccination program should be ascertained, if for no other reason than for buyer information. Information on disease control programs should be established for buyer information and the reason for those programs. The history of disease in the herd may explain the results of the BBSE examination and again be useful information to the herd owner and/or buyer.

Basic Physical Examination

For a bull to survive and function as a natural service sire (pasture or range environment), he must be able to walk, eat, see, and bear weight on his back legs. Thus, a basic examination should begin with a history, examination at a distance, and an assessment of a bull's conformation, gait, and overall appearance.

Examination at a Distance

If possible, the bulls should be observed from the fence of the holding pen while they are standing quietly. This is a good time to observe their conformation, and overall appearance. Bull identification numbers are collected for those that will need closer examination for any problems that may be seen. Move through the bulls slowly while noting any other problems. This is a good time to observe each bull's gait, possible foot issues, sight deficiencies, and bilateral symmetry. If they are being unloaded at a veterinary

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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facility for BBSE, watch them as they are being unloaded as stepping off a trailer can exacerbate lameness signs that you can check more closely. The absence of bilateral symmetry is the examiner's guide to areas of potential abnormalities.

Conformation

Selection for conformation has been influenced by judges for show purposes and is not always the conformation most desirable for durability of the bull in a pasture or range environment. Because a bull is required to cover large areas and mount and lunge multiple times in a single day, feet and legs are often the trait that determines his ability to service cattle and his longevity as a sire. Hoof and hock abnormalities lead to lameness, which affects his ability to accomplish this purpose, as well as lowering sperm quality. Screw claw, chronic laminitis, and interdigital fibromas are common. The incidence of screw claw (Figure 8.1) appears to be increasing in beef breeds and is considered heritable by the American Association of Bovine Practitioners Lameness Committee [1]. Moving up the legs, stifle and hock injuries such as collateral ligament rupture, meniscal injuries, and anterior cruciate ligament (ACL) rupture should all be investigated as they will interfere with copulation and fertility [2]. Less common and sometimes more difficult to diagnose are shoulder injuries and spinal injuries or disease [2]. These abnormalities should be recorded in the feet and legs section of the BBSE form. Defects that may be inherited should be listed in the comment block with a statement that recommends use in a terminal cross only. Research has shown that animals with shorter hoof length and greater dorsal hoof angle develop less hoof lameness [3]. Dorsal hoof angles of 50 and 55° in the rear and front hooves, respectively, appear to be the more desirable. The angle of the hock should be between 155 and 170° – this being based on information derived from the Conformation Determination System (CDS), which was a method of relating hock angles of dairy heifers with other anatomical landmarks and then correlating those metrics with the incidence of future unsoundness [4].

Examination Close Up

Once the bull enters the chute, the examination should begin with the head. The areas on and around the eyes should be examined for squamous cell carcinomas, corneal damage, and lymphomas. The nasal passages are checked for even and equal air flow, the oral cavity is examined for abnormalities, and the age of the bull is confirmed by checking the dentition (Figure 8.2). This is also a good vantage from which to observe the front feet for interdigital fibromas and the screw claw abnormality.

Examination of the Reproductive System

Scrotum and Testicles

The scrotum, testes, and spermatic cord should be examined carefully for fluid, fibrotic tissue, size, symmetry, shape, and texture. The scrotum should be examined for scars and its ability to extend or stretch to allow the testicles to cool. The testes should be palpated and have the texture of meat and be freely moveable within the scrotum. Any fibrotic or swollen areas should be noted. The epididymides should be palpated from the head dorsally, down the body,



Figure 8.1 A three-year-old Angus bull that exhibits the screw claw abnormality. This bull will require annual hoof trimming to remain sound.



Figure 8.2 Examination of the oral cavity and dentition to assess aging.

ventrally to the tail, for abnormalities such as epididymitis or fibrosis. The spermatic cord is palpated from the external inguinal ring distal to the testicle for abnormalities such as hernias, hematomas, fibrosis, or fluid. The external prepuce should be observed and palpated for abscesses and swellings, as well as hematoma of the penis directly anterior to the scrotum. Problems identified during scrotal examination can include scrotal skin lesions such as frostbite, dermatitis, scrotal edema, and blood warts. There are several normal variations of scrotal shape and testicular positioning within the scrotum. Thus, the presence of hydroceles and hematoceles as well as inguinal hernias must be ruled out in bulls with aberrances in scrotal size and shape [2, 5].

Ancillary testing utilizing ultrasound or thermography, although usually not necessary to classify a bull by the SFT standards, can be useful to further investigate abnormalities discovered during the physical examination [2].

Internal Reproductive Genitalia

A transrectal examination is utilized to evaluate the internal reproductive genitalia and can be aided or enhanced with the use of ultrasound. Each of the secondary sex organs should be carefully identified and palpated for any changes from the norm. The urethralis muscle is the first to be encountered and should be palpated for abscesses and tumors. As you palpate forward, the prostate and seminal vesicles (vesicular glands) are encountered. Palpation of both vesicular glands should begin at the bifurcation with the vesicle surrounded by the hand as it is palpated toward and to each distal endpoint. Texture, size, the presence of heat or pain, and distinction of the lobulations should be noted. Vesicular adenitis is one of the most common abnormalities suggested by the internal reproductive examination. Evidence of previous infection or injuries to the seminal vesicles can also be diagnosed by the presence of fibrosis and adhesions. Approaches to the diagnosis and treatment of vesicular adenitis are discussed in Chapter 14. The paired ampullae should be examined next for abnormalities as well as gently massaged to aid in semen collection. The examiner should use the transrectal examination as an opportunity to palpate the internal inguinal rings for size and the presence of hernias. The caudal abdomen should also be palpated for the presence of enlarged lymph nodes that could be suggestive of infection or neoplasia [5].

Penis and Internal Prepuce

The penis has already been palpated during the examination of the external prepuce but must be carefully examined and palpated while extended during the collection stage of the BBSE. Problems such as warts, hair rings, lacerations, and persistent frenulum will be discovered at this point. The internal prepuce presents itself for thorough examination during erection, allowing the opportunity to observe lacerations, warts, and fibrotic areas from old injuries that may cause deviations of the penis preventing coitus. The necessity of completely extending the penis at some point of the examination cannot be overemphasized as the previously mentioned conditions will be missed if this is not done. In-depth coverage of urogenital injuries and their diagnosis, management, and correction are covered in Chapters 15, 18, and 19.

Example of an Examination Protocol

The examination begins with a history and examination at a distance. Evaluate conformation and be certain to record observations and identification of those bulls that have issues. Watching each bull approach the chute is a good time to observe the gait of the bull and overall appearance. Once in the chute, the bull's identification (tag, tattoo, or brand ID), eyes, head, and front claws are observed and observations recorded [6]. From the side of the bull, the testicles are palpated and SC measured (Figure 8.3), the prepuce is palpated for abnormalities, and the hind feet are observed. At this point the scrotal measurement and any observations are recorded. The rear of the bull is then approached and the back, hind legs, and especially the hock joints are observed and/or palpated. Transrectal palpation is then performed, again using a constant sequence. In the pelvic area, the urethralis muscle is followed forward to the seminal vesicles, which are palpated and massaged,



Figure 8.3 The correct way to measure SC. The testes are pushed toward the bottom of the scrotum and the scrotal tape is placed at the widest area. The scrotal tape should be pulled tightly.



Figure 8.4 Transrectal palpation to evaluate the internal reproductive tract and prepare the bull for electroejaculation.

the fornix of the seminal vesicles is located, and the ampullae are palpated and massaged, as are the inguinal rings, pelvic lymph nodes, the kidney, and any viscera within reach. The ampullae and the seminal vesicles are then massaged again. The urethralis muscle and the prostate are identified and massaged while progressing toward the anus. In addition to identifying abnormalities or eliciting a painful response, which may indicate a problem, palpation and massage serves to stimulate the bull and facilitate ejaculation (Figure 8.4). Thereafter, the electroejaculator is placed on the standard program and the probe is inserted into the rectum. At this point any observation or abnormalities from palpation are recorded. As erection and protrusion occur, the internal prepuce and the penis are examined by observation and palpation for abnormalities. After microscopic examination of the semen has been done, the bull is then released from the chute. The bull is observed leaving the chute and the chute area for lameness. It is of utmost importance that a standard protocol be developed to ensure that all areas of the physical examination and SC are checked and recorded [2, 5].

Scrotal Circumference

The SC measurement is obtained while doing the general and reproductive examination [6]. It can be obtained from the side or the rear, depending on preference, facilities, and the bull's temperament. After gently forcing both testes to the bottom of the scrotum, the primary considerations are to avoid spreading the testes apart and to ensure sufficient pressure that the top surface of the measuring tape is level with the skin (Figure 8.3). Record the measurement at this point. If the circumference is below the minimum requirement, there is no need to continue the examination. Young bulls or emaciated bulls may receive a deferred classification in some cases. The SFT minimum thresholds [7] for SC are listed below:

- 30 cm at <15 months
- 31 cm at >15 to ≤18 months
- 32 cm at >18 to ≤21 months
- 33 cm at >21 to ≤24 months
- 34 cm at >24 months.

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9

Evaluation of Breeding Soundness: The Spermiogram

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Introduction

The cost of purchasing and maintaining a bull is substantial yet is easily overshadowed when a group of cycling female cattle fail to become pregnant and a complete calf crop is lost. Fortunately, this worst case scenario is a relatively rare occurrence but can have financial implications on a par with any unforeseen natural disaster. Especially in single-sire breeding systems, this should be reason enough to have a breeding soundness evaluation prior to every breeding season; however, the most costly losses associated with poor fertility are much more insidious. Profit margins in the cow-calf sector continue to shrink; therefore all producers must look for ways to improve productivity. In well-managed herds, it is expected that 95% of the breeding females will become pregnant in a 60-day breeding season, with 65% pregnant during the first 21 days. Anything less than this may be considered subfertility. Most mature beef bulls are expected to successfully mate with at least 25 cows, with lower ratios of one bull to 30, 35, and even 40 or more being used in an effort to improve efficiency while capitalizing on superior genetics. To achieve these goals, bulls must meet a set of standards below which subfertility is likely to occur.

The Society for Theriogenology (SFT) has developed a set of standards for bull breeding soundness examination (BBSE) based on available scientific data combined with input from veterinary practitioners. The SFT standards, including how the breeding potential of the bull is classified, differ almost unnoticeably from the standards adopted in other countries [1, 2]. The intent of this chapter is to provide an overview of the semen collection and evaluation in the bull. For a more indepth review, readers are encouraged to access a breeding soundness examination manual identified in the references and to review other chapters in this textbook.

Utilizing the SFT classification system, following a BBSE the animal may be classified as (i) a Satisfactory Potential Breeder, also known as "Passing" the BBSE; (ii) Classification Deferred; or (iii) an Unsatisfactory Potential Breeder. The BBSE focuses on three major categories: (i) physical soundness; (ii) scrotal circumference; and (iii) semen quality. To be classified as a Satisfactory Potential Breeder a bull must meet the described standards for all of the categories. Exceeding the standard in one category does not supersede failing the standard in another [1].

The number-one reason bulls do not receive a satisfactory classification is because of semen quality, specifically, abnormal sperm morphology.

Semen Collection

Evaluating semen quality is an integral part of a BBSE. Electroejaculation (EEJ) has been a reliable method of obtaining a semen sample for many years. Alternatives to EEJ include using an artificial vagina (AV), transrectal massage (TM) of the accessory sex glands, and aspiration of semen from the vagina of a cow following a recent natural service. Compared to these other techniques, EEJ is fast and efficient, eliminating the need for mount animals, animal training, and specialized laboratory and animal handling equipment. Nearly anyone can be trained to use an electroejaculator, and in recent years automated machines that use a preprogrammed series of electrical stimulation have been developed, enabling even the most inexperienced individual to operate with confidence. The one significant disadvantage of EEJ,

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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however, is the widely held misperception that it is painful. Due to this its use has been forbidden in several countries. [3]

In the USA, EEJ is still widely used to collect semen from bulls for presale or prebreeding breeding soundness evaluations, but as is the case with many other previously accepted management practices, the potential discomfort of animals enduring these procedures is being increasingly scrutinized. At the core of the problem has been lack of widely disseminated critical knowledge to determine if EEJ is painful and to quantify the intensity of the pain. Certainly it was known that EEJ without anesthesia was painful for humans [4, 5]; therefore it must also be painful for bulls [3]. (Editor's note: see also [6].)

The first published report on the use of EEJ in domestic animals was in 1936 [7–9]. Rams were the first animals experimented with, and although attempts to electroejaculate most species of animals have been made, the technique has been most successful with rams and bulls [9]. Electroejaculators are designed to use AC or DC power and consist of a box containing a step-down transformer to reduce the source current and a variable transformer to enable the electrical stimulation to be modified [10]. Electrical stimulation must be carried to the pelvic nerves; specifically, the pudendal nerve, located near the prostate over the pelvic urethra responsible for erection; and the hemorrhoidal nerves, located in the area of the ampullae and seminal vesicles responsible for ejaculation [9]. To accomplish this task, a solid rectal probe at least 30 cm long and at least 6 cm in diameter containing bipolar electrodes is used. Rectal probes on early models consisted of alternating, bipolar electrode strips or rings capable of providing electrical stimulation over their entire circumference. This design resulted in unnecessary stimulation of the muscles of the hindquarters reportedly causing undue discomfort and temporary paralysis in some animals [11]. This led to the development of finger-electrodes in which bipolar rings were fitted to the index and middle fingers in such a way as to avoid them coming in contact with each other. This device enabled the operator to apply electrical stimulation specifically to the urethral muscles to cause erection, followed by stimulation of the seminal vesicles to achieve ejaculation [11]. A disadvantage of the finger-electrode system was that two persons were needed to coordinate semen collection because the principal operator was unable to see if erection had occurred and when stimulation should be moved to the seminal vesicles [9].

Most probes in use today have three longitudinal electrodes. Probes with the electrodes divided into three segments have also been developed and are referred to as segmented probes. There are three caudal electrodes, three middle and two at the cranial end, and each segment can be activated as needed. Much like the finger-electrodes, the intent of this probe design is to enable stimulation of specific areas with minimal stimulation of non-target tissue [12]. The caudal segment is used to achieve erection and then the middle segment is used to achieve ejaculation. In some bulls, the activation of the cranial segment may be required to cause ejaculation [12]. The segmented probe is no longer popular due, at least in part, to its being complicated to use. In comparison with a conventional rectal probe it was shown that it was much easier to obtain full penile protrusion using the conventional probe, but ultimately both probe types were equally effective for obtaining semen emission. In a study comparing the pain response associated with the use of a conventional three-electrode probe and the segmented probe for an equal number of stimulations, and with the segments activated according to the operator's manual, no differences in cortisol or progesterone release were detected [13]. Weighted probes and probes with two longitudinal electrodes are now available. The intent of these design changes is to target the appropriate nerves with as little stimulation of surrounding tissue as possible. No critical studies have been done comparing weighted and two-electrode probes to conventional (three-electrode, no additional weight) probes; however, the clinical impressions of the author are that they are at least as effective as conventional probes yet appear to be more comfortable for the bull.

Electroejaculator probes are inserted into the rectum with the electrodes oriented ventrally and rotation of the probe is prevented by a U-shaped tail cradle or yoke. The tail cradle helps to maintain the probe in the rectum, properly oriented with the electrodes down. Until the sequence of electrical stimulation is begun it is a good idea to hold the probe in place simply by pressing the tail into its relaxed position. Horizontal or vertically (upright) oriented yokes are available. Damage to the electrical cord connecting the probe to the electroejaculator is less likely with the upright yoke in the event that bull lies down during the collection procedure, whereas horizontal yokes invariably catch on



Figure 9.1 Lane Pulsator V and two-electrode probe with vertical yoke. *Source:* Courtesy of Lane Manufacturing, Inc.

the butt bar often at the electrical connection. Upright yokes are also easy to grasp, facilitating probe extraction. Figure 9.1 shows a Lane Pulsator V and the two-electrode probe with upright yoke.

Modern electroejaculators use a sine-wave pulse at a frequency of 20–30 cycles per second [14, 15]. Other wave forms and frequencies have been tried but have been proven less effective [14]. The maximum voltage of a very common electroejaculator in use today, the Lane Pulsator IV (Lane Manufacturing, Denver, CO, USA), is 16 with a maximum current of 900 mA [12]. With this machine, ejaculation occurs with electrical impulses less than 8 or 9 V [12]; however, some bulls, usually older bulls, may require more electrical intensity to elicit ejaculation.

Electroejaculation Technique

Before attempting to electroejaculate a bull ensure that the electroejaculator is in good working order. Electrical cords and connections are most likely to be the cause if the electroejaculator unit is functioning and the probe is in place yet the bull is not responding to stimulation. Always turn the electroejaculator off before making even the slightest adjustments to trouble-shoot electrical connections. A suddenly restored connection, even if the electrical intensity is relatively low, will unduly startle the bull. The rectal probe selected should be of sufficient diameter to enable full contact with the rectal mucosa, but not so large as to prevent its insertion. Usually, a probe with a diameter of 6.5–7.5 cm will suffice. Very large (>900 kg) or older animals may require a 9-cm probe to ensure contact with the rectal mucosa [16]; 6- to 6.5-cm yearling probes are available and are much easier to use on smaller bulls [17]. Bulls must be suitably restrained to prevent side–side and back–forth motion and should not be handled in such a way as to cause excitement. Overly excited animals tend to vocalize more during the procedure [18] and are more difficult to collect [16]. Cattle squeezes generally work very well for BBSE provided there is enough room for larger bulls to fit into them. Earlier models were designed mainly for use with cows and often are not long enough to accommodate a mature bull. A post, often referred to as a butt bar or kick post, placed horizontally across the chute immediately behind the bull will prevent the bull from moving backward or kicking the examiner. Ideally, the butt bar should be placed immediately behind the bull so that any kick action is softened, yet at a level that enables examination of the scrotum and testes. Modern hydraulic squeezes are the author's preferred system for examining bulls. Side–side pressure is infinitely variable and easy to apply, virtually eliminating any kicking. The side–side squeeze pressure and squeeze pressure on the neck must be relaxed prior to beginning the EEJ process. Failure to obtain a semen sample, bulls lying down in the chute, and poor quality ejaculates have all been caused by excessive squeeze pressure. Giving bulls room to move and to get their feet placed under them will go a long way toward shortening the duration of the EEJ procedure and yielding good quality samples.

A per rectum examination of the bull's ampullae, seminal vesicles, prostate, pelvic urethra, and inguinal rings should be conducted accompanied by a thorough clean-out of feces from the rectum. Massage of the pelvic urethra and accessory sex organs lasting 30–60 seconds will provide sexual simulation for the bull and relaxation of the anal sphincter to enable insertion of the probe. An elbow to wrist, back and forth rectal massage with immediate insertion of the probe following arm removal works best, coupled with the smallest probe that is still effective for the size of bull being collected. The smaller yearling probes have proven effective in at least 9 out of 10 mature bulls when matched with well-designed, late-model electroejaculators. Lubrication of the probe with palpation sleeve lube or even water will facilitate insertion and enhance contact with the rectal mucosa [16].

Electroejaculators available today are similar in function with features that appeal to a variety of users. Advances in electrical circuitry and battery technology have been adopted by manufacturers, resulting in enhanced portability without the need for a constant electrical supply. Machines available today may be used to electroejaculate over 100 bulls on a single charge (Bob Green, Lane Manufacturing Inc., personal communication). A significant innovation has been preprogrammed, automated EEJ sequences that increase electrical voltage in a step-wise fashion, bridging the gap between the art and science of collecting a bull. Manual control of voltage intensity is still available via a rheostat knob and step-wise control buttons, allowing the operator to use the machine according to personal preferences. Experience is a great teacher when collecting semen from bulls using EEJ. Effective stimulation sequences vary with the breed, age, size, temperament, handling system, weather, and individual animal. For the novice a great deal can be learned by using the preprogrammed sequences, then try to emulate a sequence using the manual controls. Learn to watch the response of the bull not the indicator systems on the machine. The initial application of electrical stimulation must be applied carefully while observing the response of the bull. The operator should increase the electrical intensity just enough to cause a slight contraction of the muscles of the hind limb. Thereafter, with each successive stimulation the intensity of the voltage should be increased slightly and should last 1–2 seconds followed by ½–1 second of rest until erection, then ejaculation occurs [12, 16]. Some bulls are more difficult to collect than others for a variety of reasons. Common

problems are inadequate contact between the probe and the rectal mucosa (excess feces, small probe, or a buildup of dried mucus or lube on the electrodes); insufficient voltage; bulls that have been recently used for breeding [16]; and excessive squeezing pressure on the neck or sides of the bull. Even when these potential problems are addressed, EEJ may still be unsuccessful with a small number of normal, fertile bulls.

Semen collection handles are useful for safely extending the operator's reach to obtain a sample. Disposable, single-use, funnel-shaped collection cones have largely replaced reusable latex rubber cones and are designed so that the wide end can be spread open inside the cylinder of the collection handle and held in place with a rubber ring on the outside of the cylinder. Commercially available semen vials or a urinalysis tube can be passed, bottom end first, through the collection cones and pulled snuggly through the smaller opening to make a tight seal near the opening of the vial (Figure 9.2). Bovine semen is sensitive to chilling, with the most noticeable effect being a rapid decline in sperm motility. Vials and collection cones should be kept in a warming area and only brought out just prior to use. Spare vials can be kept warm in a chest pocket or on a microscope warming stage. Warming jackets containing water can be fashioned from plastic bottles and attached to the collection handle using adhesive tape. Styrofoam coffee cups can also work well as a collection vial, with their insulating capability being an additional benefit when ambient temperatures are cool.

Numerous anesthetic protocols have been employed to evaluate and potentially reduce potential pain or anxiety associated with EEJ, but none of them can be considered to be viable alternatives to conventional EEJ. Caudal epidural anesthesia with 2% lidocaine appeared to be just as effective as conventional EEJ with lesser elevations in cortisol, but did not significantly affect heart rate change [13, 19]. The benefits did not appear to justify the time and effort expended administering the epidural. Xylazine, administered in an epidural or intravenously was effective for reducing pain, but because of associated ataxia and recumbency its use is not practical [12]. Intrarectal lidocaine reduced intrarectal smooth muscle contractions, but its effect is only superficial as heart rate change remained unchanged compared to conventional EEJ [3]. Although not all studies agree, the majority have shown that EEJ can be mildly painful [12] and stressful to bulls; however, heightened awareness and improvements in probe and electroejaculator technology coupled with careful, conscientious use have substantially decreased these concerns.



Figure 9.2 Semen collection handle, cone, and vial.

A mainstay in bull studs, the AV and a mount animal are very useful for collecting semen from trained animals, but are not practical for use with most beef bulls used for pasture breeding. Phantom cows, similar in design to the phantoms used for stallions, complete with an AV mounted within the frame were tested with untrained beef bulls. Not even the application of estrus cow urine sprinkled on the hides that covered the phantoms would entice the bulls to mount and serve [12]. Serving capacity tests are used to evaluate libido and the ability to mount and complete service [20–22]. These tests are conducted by introducing one or more bulls into a pen containing at least one cow restrained in stocks. Having cows in estrus may increase the sexual interest of some bulls [20]; however, willingness to stand to be mounted is a major cue used by breeding bulls that the female is ready to be mated. Therefore because they are restrained the cows used in these tests do not need to be in estrus. Although serving capacity tests are not considered to be reflective of true breeding behavior in juvenile bulls, most mature bulls will express sexual interest in and mount a restrained cow [20].

Early attempts to modify the serving capacity test to enable the collection of semen involved having a person deflect the bull's penis away from the cow and into an AV. Most untrained bulls were unwilling to mount with a person standing close by, so attempts were made to conceal the operator behind drapes while the bull mounted and semen was collected [12]. High libido bulls were undeterred by this system, but medium to low libido bulls seemed to be distracted by the drapes or the presence of humans behind the drapes and refused to serve. [12]. The next modification was to design an AV that could be fitted inside the cow's vagina, called the internal artificial vagina (IAV). The IAV consisted of a wire frame supporting a 7.5-cm length of rubber tubing with a plastic specimen bag attached to the end placed closest to the cervix [23]. Once the bull had mounted the cow and completed service the IAV could be quickly removed and the semen sample recovered. Over a two-year period, the IAV was compared to EEJ as a method of collecting semen from 165 range bulls. Fifty four and

seventy percent of the bulls served the IAV in years one and two, respectively versus a 100% success rate when EEJ was used. Five percent of the bulls were found to be unable to serve cows due to physical abnormalities. Bulls that were unwilling to serve the IAV in year 2 were given a second opportunity, but the result remained the same. Over the subsequent breeding season, the breeding behavior of 15 of the bulls that did not serve the IAV was compared with 15 that did. Most of the bulls that would not serve the IAV were observed breeding cows but mounted less frequently than their counterparts [23]. The IAV proved useful for identifying bulls with physical abnormalities but was not a viable substitute for EEJ. Other concerns included the spread of venereal disease and the welfare of the restrained mount animals [12].

Transrectal massage of the seminal vesicles, ampullae, and pelvic urethra has been used as a method of obtaining a semen sample but is not as reliable as EEJ. In one experiment, TM was shown to be nearly as effective as EEJ for obtaining a semen sample [24], but the percentages of motile and live sperm were less than those of EEJ samples. This was attributed to the tendency of massaged bulls to dribble semen slowly, thereby exposing the sperm to cool temperatures for a longer period of time [24]. Two more experiments were conducted comparing TM with EEJ in infrequently handled, mature breeding bulls (range bulls) and yearling bulls. In an attempt to prevent chilling of the sperm, semen collection tubes were suspended in a 37 °C water jacket during the collection. Despite this effort, fewer motile and live sperm were recovered from TM samples in the range bulls, but there was no difference in percentages of motile sperm in the yearling bulls [25]. These differences were attributed to lack of penile protrusion during semen collection with TM – a problem that was particularly evident with the range bulls [25]. The differences in live and motile sperm percentages were not large, but these authors cautioned that poor semen sample quality and an inability to examine the penis could jeopardize the quality of a BBSE [25]. Transrectal massage of the seminal vesicles and ampullae can be useful following an unsuccessful attempt to electroejaculate a bull. In these cases, the maximum voltage has been reached but only preseminal fluid has been emitted. One useful trick is to rest the bull for at least 30 seconds and restart the collection at a much lower setting. If this quick fix fails, then the probe can be removed and TM may be effective.

Aspiration of semen from the vagina of a recently bred female is a last resort method of obtaining a sample as it is time consuming and requires a female in estrus that must be restrained to allow a sample to be obtained. A syringe and infusion pipette will facilitate the collection of mucus and semen from the anterior vagina.

Semen Evaluation

Once the semen sample has been collected the vial can be easily detached from the semen collection cone. To avoid spillage it works best to pull the cone sideways and slightly downward away from the vial, which has the effect of stretching and shearing the cone off on the edges of the vial. Always keep the semen vial protected from the cold prior to evaluation. A simple and effective method is to hold the vial in a warm hand with the fingers completely encircling it. If evaluation is not going to occur right away the sample should be transferred to a 37 °C water bath. The chest pocket of a close-fitting pair of coveralls has also worked well for short-term storage, especially if the hands are needed for other tasks.

Although not always recorded, the skilled examiner should know how to assess each of the following when examining a semen sample:

- Color and consistency
- Volume
- Concentration
- Gross motility
- Individual motility
- Sperm morphology
- Percentage staining alive

Color and Consistency

A good quality ejaculate should be opaque, creamy-white in color. A yellow, buttery hue may also be seen and is a variation of normal when it occurs in concentrated samples with no apparent aberrations. The yellow coloring may be associated with riboflavin in the diet [26] and is different from a yellow tinge that may be evident with urine contamination. A urine-contaminated semen sample will usually be variably diluted and will have an odor of urine. (Editor's note: if unsure, utilization of a "BUN strip" [blood urea nitrogen] will confirm or rule out.) Preseminal fluid is clear, watery, and odorless. Semen samples diluted with preseminal fluid will not be opaque but rather have a degree of clarity on a spectrum, comparable with skim-milk, watered-down juice, or even clear fluid. Generally, preseminal fluid emission is noted during the EEJ process, but it is not collected in favor of waiting for the appearance of the sperm-rich fraction. Especially in difficult collections, a variable amount of preseminal fluid will be collected, thereby diluting the sample. A pink or red hue or even streaks of red in the semen sample are usually indicative of blood contamination. The source of the blood should be addressed. Blood-contaminated samples are common when semen

collection follows preputial scraping for *Tritrichomonas foetus* and *Campylobacter foetus*, especially if penile protrusion did not occur during EEJ. Clumps of white cellular material in the vial are highly suggestive of a high neutrophil content.

Volume

Always note the volume as it is an indication that a representative sample was collected; however, volume is influenced by the collection process and is therefore not a relevant measure. In other words, the volume of an EEJ-derived semen sample should not be compared with ejaculates from the same or different bulls. The one exception is in cases of sperm accumulation, the "rusty load phenomenon," where 25–40 ml of concentrated semen may be collected easily during a single ejaculation [2]. A typical EEJ-derived semen sample is 1–5 ml.

Concentration

A subjective evaluation of concentration should be made by examining the tube containing the semen to ensure that a representative sample has been obtained. A poorly concentrated sample will be watery and translucent containing <250 million sperm per milliliter, whereas a concentrated sample, typical of a mature bull, will appear creamy and grainy when examined against a dark background containing at least one billion sperm per milliliter. Very concentrated, high-volume ejaculates occur in cases of sperm accumulation. A watery, poorly concentrated sample from a mature bull is typical in cases of testicular degeneration. Closely linked to volume, the concentration of a semen sample also varies with the collection technique. EEJ is not an appropriate method of determining the sperm-producing capability of a bull.

Gross Motility

Gross motility or gross wave motion is influenced by the percentage of progressively motile sperm, the sperm concentration, and the vigor or rate of speed of the motile sperm. Determining gross motility is a fast and simple method requiring just a drop of semen on a microscope slide which is then viewed using bright field microscopy at 40–125× magnification [2]. Vigorous swirls and eddies are indicative of a concentrated semen with a very high proportion of vigorous motile sperm. A depression affecting one or more of the three factors will, in turn, have a negative effect on gross motility. For example, sperm motility may be very high in a poorly concentrated semen sample yet the gross motility appears poor. Another common occurrence is when a highly concentrated sample becomes chilled, negatively affecting sperm vigor. As a result, gross motility should be interpreted carefully, leaving individual motility as a more reliable method of assessing sperm motility.

Individual Motility

Individual motility or more precisely percentage of progressively motile sperm is determined by placing a 2- to 4-mm drop of semen on a clean microscope slide over which a cover slip is dropped in place. The seminal fluid should spread just to the edge of the cover slip to form a thin layer in which the sperm can be viewed within the same focal plane [2]. Too much semen and the cover slip will float, making visualization of individual sperm more difficult, and very concentrated samples may be impossible to evaluate without dilution. Although readily available, the low pH of physiologic saline can diminish sperm motility. Warmed sodium citrate solution [17], commercial semen extender, or even fresh sperm-free seminal fluid are better choices to use as a diluent [2]. Individual motility is recorded as a percentage, with minimum acceptable proportions of 30% [1] and 60% [2] being used by the respective SFT and Western Canadian Association of Bovine Practitioners' guidelines. Several fields should be examined at 400× magnification, observing the proportion moving forward versus those that are immotile, or barely motile, and an average of the percentage motile sperm can be estimated, usually rounded to the nearest multiple of 10. Individual sperm motion is most effectively observed using phase contrast microscopy. Most modern microscopes can be fitted with phase contrast for a modest cost. Sperm motility can be easily affected by heating, chilling, and contamination with urine or other fluids, soaps, etc. A finding of poor motility should be substantiated by sperm morphology and vitality (percentage staining live) before it is considered reflective of fertility. If a finding of poor motility cannot be substantiated then a second sample should be evaluated.

Sperm Morphology

An Assessment of sperm morphology should always be performed. In most cases, irregularities noted with one or more of the other parameters will be evident in the sperm morphology assessment. The minimum percentage of morphologically normal sperm is 70.

Percentage Staining Alive

Percentage staining alive requires the use of a vital stain (see section "Preparation of Semen Smears") wherein eosin will cause the dead sperm to stain pink. The percentage

staining alive is a good indicator of sperm vitality which will be positively correlated with progressive motility when semen handling and slide preparation have been performed correctly. Example reasons that the percentage staining alive may be high but motility is poor could occur with midpiece/principal piece defects or if there has been urine or other chemical contamination of the semen sample.

Preparation of Semen Smears

Evaluation of sperm morphology begins with a well-prepared smear. Eosin-nigrosin is the most recommended and widely used stain in use for evaluation of bull sperm. Eosin penetrates damaged sperm membranes to stain dead and non-viable cells pink, hence it is referred to as a vital, or vitality, stain. Nigrosin provides a dark, purple background, enhancing the appearance of both live and non-viable cells. Eosin-aniline blue is another vital stain that is less popular nowadays. Aniline-blue (blue color) takes the place of nigrosin (purple color) as the background stain. Live sperm do not take up the eosin stain and appear white; half-stained sperm are protected by the acrosomal membrane covering the anterior end, but eosin is able to penetrate the non-acrosomal membrane distal to the equatorial region. These half-stained sperm are more common in semen smears prepared under less than ideal conditions such as cold ambient temperatures. They are non-viable, but in the opinion of the author they are iatrogenic in origin versus the entirely pink sperm that was non-viable or dead for a sufficient period of time before staining to lose or suffer damage to the acrosomal membrane. When semen samples are handled correctly and there are no morphologic defects affecting sperm motility, the proportion of sperm staining live and the percentage of progressively motile sperm should be close to the percentage of live sperm, being approximately 5–10% higher.

Non-vital stains such as modified Wright Giemsa (Diff Quik) and India ink provide a dark background, but do not have a cell wall-penetrating component. They may be used to evaluate sperm morphology but should be used only if eosin-nigrosin is not available. Diff Quik is a superior stain for definitively identifying white blood cells, displaying the characteristic bean-shaped nucleus of neutrophils very well (Figure 9.3). Neutrophils appear as large, white, circular structures, often one and a half to two times the size of a sperm head when viewed on eosin-nigrosin stained smears (Figure 9.4). Diff Quik is a superior stain for definitively identifying white blood cells. A distinct advantage associated with the use of eosin-nigrosin is the rapidity with which slides can be prepared and evaluated. Stains that require long periods of air drying and several steps not only are time consuming but also can result in the loss of

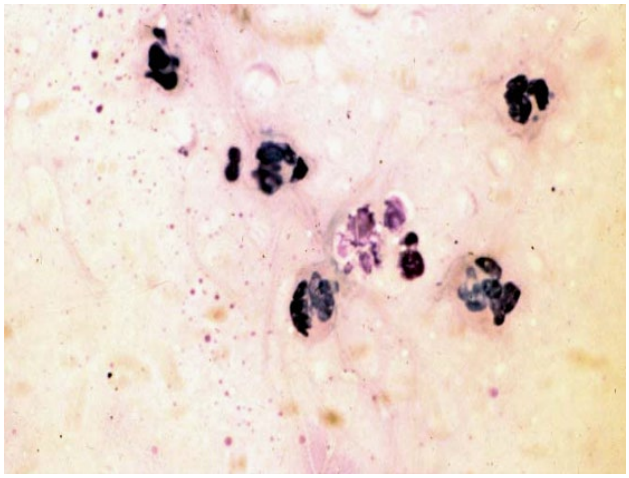


Figure 9.3 White blood cells (neutrophils) on a Diff Quik stained smear. Note the faintly stained sperm.



Figure 9.4 White blood cells (wbc), a speroid cell (sc), detached head (dh), a shed droplet (sd), and distal midpiece reflexes (dmr) on an eosin-nigrosin stained smear.

cells. Unstained wet mounts should never be the sole means of evaluating sperm morphology – the ability to identify many sperm defects is inadequate. Wet mounts are useful for observing the unusual motility patterns of live sperm with midpiece defects and for verifying whether a perceived staining artifact is real or not.

Prior to beginning the evaluation of a semen sample, clean slides should be warmed on a warming stage for several minutes to prevent any artifactual, cold-related changes. To prepare a semen smear, place a 4- to 5-mm drop of stain at one end of the slide, adjacent to the frosted end if applicable. Frosted slides are ideal for labeling, which will facilitate the filing of semen smears as part of the medical record. Stain should always be placed first to limit exposure of the sperm to the damaging effects of chilling and desiccation before fixing with stain. Place a slightly smaller drop of semen adjacent to the stain and immediately mix the two together.

Lifting the slide off of the warm stage with one hand and mixing with the other hand is far less awkward then leaving the slide on a flat surface. Wooden applicator sticks (Applicators, Puritan Medical Products Company LLC, Guilford, ME, USA) work well for obtaining droplets of semen and mixing. Using the stir stick, spread the mixture down the length of the slide in a stopping and starting fashion which will effectively create thick and thin areas of stain (Figure 9.5). This will help to provide the ideal area to count approximately 10–20 sperm per microscope field once the slide is dry. Another way to prepare a smear is to first mix the semen and stain as described above then use a second microscope slide to make the smear, similar to making blood smear. A better result will be achieved if the second slide is pulled back slowly, not rapidly as is the recommended practice when preparing a blood smear. Smears should be dried as quickly as possible to prevent the occurrence of bowed tails, an artifactual occurrence due to the hypotonic nature of eosin-nigrosin. This may be accomplished by returning the slide to the slide warmer and by blowing on the slide to speed drying.

The Feulgen staining technique is a multistep procedure that begins with an air-dried smear. The exposure of the dried sperm cells to hydrochloric acid exposes aldehyde groups in the DNA which then bind with the Schiff's reagent, resulting in magenta staining of the DNA [27]. Feulgen staining is an excellent way to augment eosin-nigrosin stained smears, particularly when abnormal DNA condensation is suspected or to get a more accurate differential count of the number and type of nuclear vacuoles.



Figure 9.5 Semen smear preparation.

Classification and Evaluation of Sperm Morphology

Differential counts of sperm morphology are done with oil immersion, bright field microscopy at 1000× magnification. Always use immersion oil labeled for microscopy. Other oils, e.g. mineral oil, have a different refractive index and may penetrate the objective and effectively ruin it. Blood cell counters relabeled for use as sperm cell counters are an invaluable tool for the practitioner who evaluates more than a few bulls each year. At the Western College of Veterinary Medicine we label keys by the location of the defect on the sperm as shown in Table 9.1.

An image of a labeled sperm cell counter is shown in Figure 9.6. "Detached" or "loose" refers to detached heads. Detached heads with abnormalities, for example, the pyriform shape or nuclear vacuoles, should be counted as "detached abnormal." Heads without abnormalities are counted as "detached normal." Every sperm within a field should be counted to avoid bias. Exceptions will be if clumps of sperm are present obscuring other sperm, or if there are simply too many sperm preventing the complete inspection of individual cells. Fields should be inspected in systemic fashion – left to right, top to bottom has been an effective method for the author.

Differential counts are reported as "defects per 100 cells." Each time a key is pushed a cell is counted and added to the total. In cases where a sperm cell has more than one defect the respective keys should be depressed simultaneously. The two defects are counted, yet only one cell is added to the total. When the counter reaches 100 cells a bell is sounded. Counting just 100 sperm cells will be sufficiently representative if just a few abnormalities are recorded. When many abnormalities are encountered it is advised to count at least 300 cells to improve the reliability of the morphology assessment. Reliable counts should not differ by more than 10%, or, in other words, 10 cells. When reviewing my counts, I generally disregard any outliers in favor of completing another 100-cell count. Once satisfied that my counts are representative I will report the average of the suitable counts.

When sperm morphology is examined, several questions may arise about the specific type of defects observed:

- Is it an aberration or a significant abnormality?
- What would be the effect on fertilizing ability (sperm transport, binding to the oocyte, oocyte penetration, zygote formation)?

Table 9.1 Layout of an example label for keys used by the Western College of Veterinary Medicine, Saskatoon.

Head	Midpiece	Principal piece	Detached (loose) abnormal	Detached (loose) normal	Proximal droplet	Acrosome (other)	Normal



Figure 9.6 Cell counter with keys labeled for sperm cell morphology.

- What would be the tolerable level for bulls in natural service, or semen used in AI?
- What are the implications for the bull (cause, prognosis)?

Readers of this chapter are encouraged to review sperm structure and spermatogenesis to develop a more complete understanding of sperm morphology (see Chapter 3). Classification systems have been developed to try to simplify semen evaluation; however, there appears to be quite a lot of misunderstanding of them. The earliest system used was the primary/secondary sperm defect system. By definition, a primary defect is one that originates during spermatogenesis – within the testicle – and a secondary defect is one that originates within the epididymis [28]. All head defects, such as knobbed acrosomes (KAs), microcephalic sperm, pyriform heads, and nuclear vacuoles, would thus be primary defects. Some tail defects would be primary, but others, notably the Dag defect and the distal midpiece reflex defect, develop in the epididymis and would thus be secondary defects. However, if these defects developed in the epididymis because of a weakness in structure that occurred in spermatogenesis they might be defined as primary defects. Following this line of reasoning there would then really be very few secondary defects. Distal midpiece reflexes and proximal droplets appear to develop in sperm that were structurally normal upon entering the epididymis. However, it could be argued that sperm with malformed heads or tails are more likely to retain a cytoplasmic droplet in the proximal position. The supposed origin of a defect bears little relevance to the effect of the defect on fertility and probably is only useful for determining when a defect might appear in the spermiogram following an insult to spermatogenesis.

The "major and minor" system of sperm morphology classification was created to remove some of the confusion associated with the primary/secondary sperm defect system. In this system a major defect was a sperm aberration which had been associated with infertility. Minor defects were those that had not been shown to be associated with infertility [29, 30]. Over time, more evidence has been gathered, including the reporting of defects not included in the original list. Sperm aberrations that were categorized as minor have been found to have a significant effect on fertility. In defense of the early work, it would seem that at that time very few bulls had been found that produced high numbers of some of these defects. It is notable that two sperm aberrations that were listed as minor defects, abaxial tails and distal droplets, have been shown to have no effect on fertility and should be disregarded when classifying sperm as normal or abnormal.

Use of the primary/secondary and major/minor sperm defect classification systems has been replaced by the differential count of sperm defects system described previously, where the predominant defects are listed as a percentage and the effect on fertility is determined based on the current knowledge of each type of defect.

A system of classifying sperm defects that has proven useful in predicting the effect on fertility is the compensable/uncompensable system. Based on the understanding that a certain population of live, motile sperm must travel to the site of fertilization and that some abnormal sperm can penetrate the ovum while others cannot, the compensable/uncompensable system has been particularly useful for determining sperm concentrations per insemination dose for the artificial insemination industry. Sperm with impaired motility that are unable to travel to the site of fertilization, or that can travel but cannot penetrate the ovum, can be compensated for by motile sperm – compensable defects. Defective sperm, which are able to penetrate the zona pellucida and initiate the zona block are uncompensable [31, 32]. Noteworthy examples of uncompensable sperm are those with diadem vacuoles or abnormal DNA condensation. Although fertilization by a sperm with an uncompensable defect is equally as possible as a normal sperm, the risk of early embryonic loss is much greater with the uncompensable sperm defect.

As mentioned previously, differentially counting sperm defects to produce a spermiogram is a much less confusing approach to assessing sperm morphology. There are approximately 25 recognized sperm abnormalities – some are artifactual and some have no effect on fertility. Table 9.2

Table 9.2 Listing of recognized sperm defects.

1) Pyriform heads
2) Tapered heads
3) Microcephalic/macrocephalic heads
4) Vacuoles – diadem, single vacuoles, confluent vacuoles
5) Clumped DNA
6) Detached (loose) heads – normal, abnormal
7) Decapitated defect
8) Rolled-crested-giant head syndrome
9) Teratoids
10) Knobbed acrosome – beaded, flattened
11) Ruffled and detached acrosomes
12) Distal midpiece reflex
13) Mitochondrial sheath defects
14) Dag defect
15) Stump tail defect
16) Corkscrew defect
17) Pseudodroplet defect
18) Coiled principal piece
19) Proximal cytoplasmic droplets

lists 19 defects; however, some defects have been grouped together. Examiners should regularly review abnormal sperm types. Comparing differential counts with colleagues and experts in sperm morphology are good ways to ensure that one is performing consistent, quality evaluations. Taking the time to compare a single sperm with a questionable appearance with images of known defects is an effective way to learn.

A good quality, well-maintained microscope and some knowledge on how to optimize image quality are important. Oil easily captures dust and debris, so it is good practice to clean the oil immersion objective regularly. A solution of 70% ethanol on a lens-appropriate cloth works well.

Sperm Abnormalities

Several of the most common or noteworthy sperm defects are presented below.

Normal Sperm

Nearly all novice examiners will be able to recognize a normal sperm at first glance, but if questioned they will be unsure. An effective technique is to develop a system so that each sperm is thoroughly examined. Normal sperm vary in shape both within a single smear and more so between bulls. Heads may appear short and wide or elongated and narrow. Sometimes a sperm head may look excessively narrow, but if there are no other, more obvious, disturbances in head shape and most of the sperm in the ejaculate appear similar then these sperm can be considered to be normal (Figure 9.7).

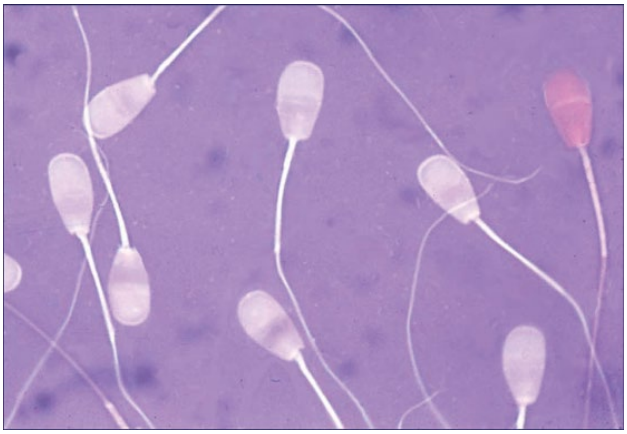


Figure 9.7 Normal sperm stained with eosin-nigrosin. The dark staining sperm is dead.

Sperm Morphology – Minimums and Maximums

The widely accepted minimum proportion of morphologically normal sperm is 70%. Below this threshold it is expected that fertility will be impaired and a bull cannot be classified as a satisfactory potential breeder. Generally, bulls that are at or near this threshold have at least two or three sperm defects; however, as a precaution, it is recommended that the proportion of sperm with nuclear defects or proximal droplets should not exceed 20% and the proportion with acrosome or midpiece abnormalities should not exceed 25%.

Artifacts and Debris

Commonly found artifacts include bowed midpieces (also called bowed tails) and bent tails (also called simple bent tails and hypotonic shock). Neither of these anomalies should be counted as abnormal in the differential count. Bowed midpieces are easy to recognize as rainbow-shaped, C-shaped, or U-shaped. A key characteristic is that the tails (midpiece and principal piece) do not fold as is the case with the distal midpiece reflex. The occurrence of bowed midpieces is associated with faulty slide preparation, most often when slides and the stain are cold. Examination of motile sperm on a wet mount can be used to confirm that this aberration is artifact [27].

Hypotonic shock is usually manifested as terminal whipping or looping of the principal piece and is caused by prolonged exposure to hypotonic stains (eosin-nigrosin and eosin-aniline blue) before drying. Drying is often prolonged when stains and slides are cold. The looping may appear similar to the distal midpiece defect (DMR) except that

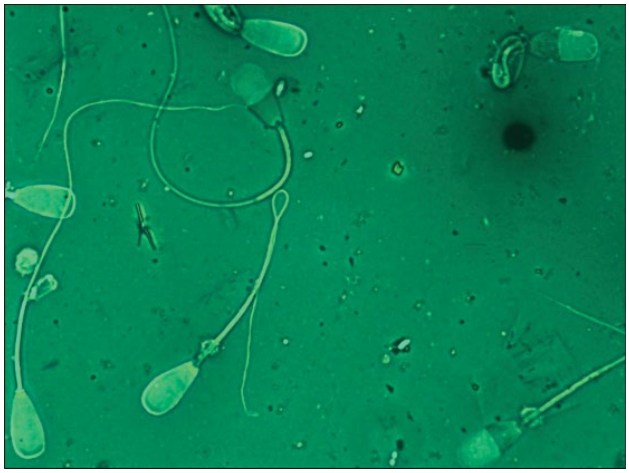


Figure 9.8 A sperm with a bowed midpiece and a sperm displaying hypotonic shock (looped tail).

there will be no trapped droplet material. Closely examining the terminal portion of the midpiece and comparing the aberrant sperm with other sperm on the smear will facilitate classification (Figure 9.8).

Other debris includes white blood cells, shed droplets, and spheroid cells (shown in Figure 9.3) as well as epithelial cells, bacteria, and red blood cells.

Morphological Aberrations that Are Counted as Normal Sperm Cells

Arguably, sperm cells displaying a distal cytoplasmic droplet or abaxial tails are not normal; however, the presence of very high proportions of either of these aberrations has not been shown to negatively impact fertility. Distal droplets are shed after a period of exposure to vesicular fluid and will often be seen as tiny white circular structures on the smear (Figure 9.3). If there are no other defects visible, sperm displaying a distal droplet or an abaxial tail should be counted as normal (Table 9.3).

Head Defects

Pyriform and Tapered Heads

Pyriform (pear-shaped) heads are commonly recorded in spermiograms, usually with other sperm defects. The pyriform shape is very distinctive, with varying degrees of severity. The apex of the sperm head is very rounded and the sides are sharply tapered all the way to the base of the sperm head. Many severely pyriformed heads are much smaller overall than normal sperm. Appearing narrow from the apex to the basal plate, truly tapered heads are not as common as pyriform heads. A good rule of thumb is that if the examiner must wonder if a sperm is tapered or not, then it probably is not. Tapered heads and pyriform heads frequently appear together in semen smears. As mentioned in the discussion of normal sperm, it is quite common for individual bulls to produce sperm with narrow heads. Studies have shown that moderately narrow sperm heads do not have any effect on fertility; however, the fate of pyriform heads depends on the severity of the aberration. Severely misshapen pyriform heads are not capable of fertilization and are likely compensable. Mildly pyriformed heads, on the other hand, are capable of fertilization but with a much greater odds of embryonic loss. Pyriformed and tapered heads often appear with other sperm defects within a few weeks following a heat- or stress-related insult to spermatogenesis [2] (Figure 9.9).

Table 9.3 Abnormal sperm morphologies, artifacts, and other cells not included in a sperm morphology differential count.

Morphology	Artifact	Other cells
Abaxial tails	Bowed midpiece	White blood cells
Distal droplets	Simple bent tail (hypotonic or cold shock)	Medusa cells
		Spheroids
		Epithelial cells

Nuclear Vacuoles

Vacuoles arise from invaginations into the inner nuclear membrane that extend into the sperm nucleus. On eosin-nigrosin stain smears, vacuoles appear as dark areas on the sperm varying in size and location according to their type. Small pale or white-colored spots are occasionally seen that are most often shed distal droplet material or other artifact. There are three types of vacuoles: (i) diadem vacuoles (Figures 9.10 and 9.11); (ii) apical vacuoles; and (iii) large confluent vacuoles (Figure 9.12). Diadem vacuoles are the most common type, appearing as a partial or complete arrangement of vacuoles on the equatorial region of

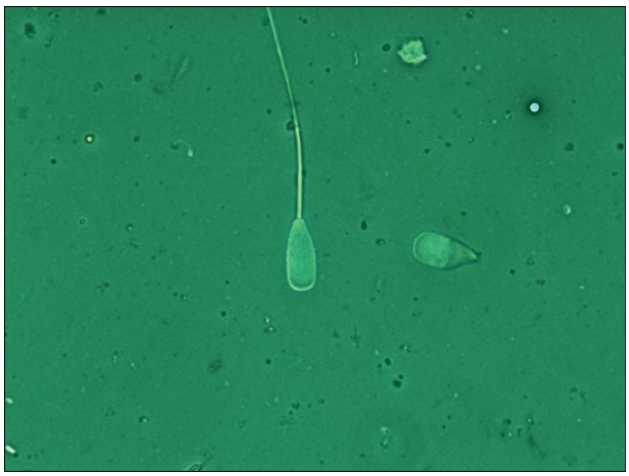


Figure 9.9 Tapered head and a detached, pyriform head.

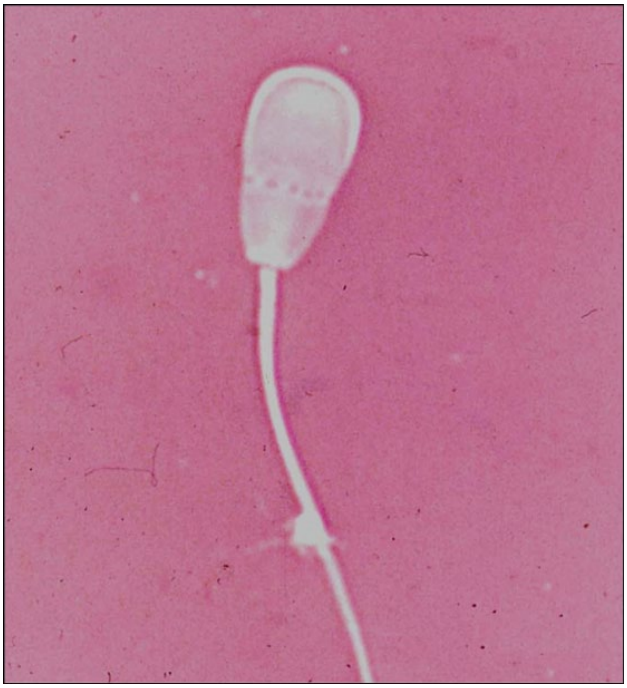


Figure 9.10 Sperm with diadem vacuoles (presentation 1).

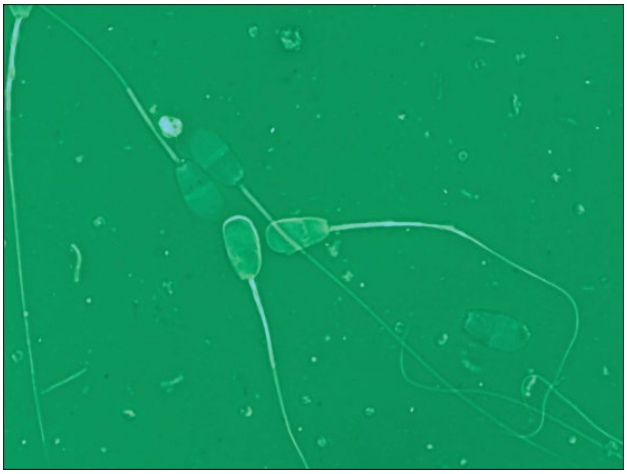


Figure 9.11 Sperm with diadem vacuoles (presentation 2).

the sperm. When the vacuoles form a complete line across the sperm the arrangement has the appearance of a necklace, hence the name diadem which means diamond necklace. Apical vacuoles occur as single or multiple small vacuoles appearing most commonly in the apex of the sperm head. Apical vacuoles may occur alone, affecting few to several sperm, but most often appear in spermiograms with diadem vacuoles. Confluent vacuoles are the most easily visualized of the vacuoles as they are made up of several vacuoles that become joined together, usually distorting the shape of the sperm head.

Diadem and apical vacuoles are easily missed on eosin-nigrosin smears. Clean objectives, a quality microscope,

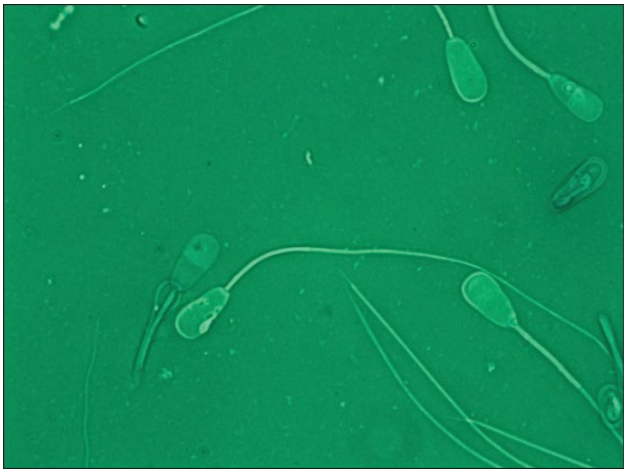


Figure 9.12 Sperm with confluent vacuoles and sperm with distal midpiece reflexes.

good quality smears, and close examination of the sperm heads looking specifically for vacuoles will improve the examiner's success. Feulgen stained smears are superior for identifying nuclear vacuoles where they appear as pale circles (diadem and apical) or large pale areas (confluent) on the sperm heads. Oftentimes if a few vacuoles are noted on an eosin-nigrosin stained smear, many more will be seen on the Feulgen stained smear.

The effect of apical vacuoles on fertility is inconclusive, whereas there is substantial evidence condemning the diadem as a significant cause of infertility. The diadem vacuole is not a compensable defect. Studies evaluating the effect of purely large confluent vacuoles on fertility are limited, but available information does indicate that fertility is impaired by a high proportion of these vacuoles being present [2].

Stress, heat, toxins, and genetics have all been proven or suspected to be associated with the appearance of diadem vacuoles in bull sperm. Some bulls have been shown to have variable proportions of diadem vacuoles in their spermiograms throughout their lives, while others have small numbers that only appear following a stressful event. Bulls having modest to large numbers of sperm with diadem vacuoles should be considered suspect and at the very least monitored for several months to see if the condition resolves. In the opinion of the author, this may occur with some bulls following the resolution of a stressful event, but there is no certainty the problem will not reoccur sometime later in life.

Microcephalic and Macrocephalic Heads

Sperm heads that are clearly smaller or larger than others are called microcephalic or macrocephalic (Figure 9.13), respectively. Representing a mistake made during spermatogenesis, they are believed to contain either too little or too much nuclear chromatin. These sperm are not believed to



Figure 9.13 Macrocephalic sperm with double tail (center).

be capable of fertilization themselves, but when they are present in a spermiogram they account for no more than 2–5% of the differential and are probably compensable.

Knobbed Acrosome

The most common morphological aberration affecting the acrosome is the KA defect. There are two forms of this defect: indented and beaded (Figure 9.14). The indented form is the most common presentation, showing up in spermiograms from a few to most of the sperm being affected. Instead of having the gentle convex curve that typifies the top or apex of the head, sperm with indented KAs will have a flattened to indented apex and variable thickening of the acrosomal ridge. A smaller proportion of sperm will not show the flattened apex but instead have a small white (eosin-nigrosin stained smear) semicircular structure just below the apex. These sperm often serve as an indication that the other sperm should be carefully scrutinized to look for flattening and indentation. A few sperm with the indented form may be noted in spermiograms with other defects and have been shown to appear three weeks following a thermal (heat) shock to spermatogenesis [33]. When differential counts showing 30%, 50%, or greater proportions of KA are present the bull should be considered suspect and further investigation is warranted. The indented form occurs in several breeds of cattle. To date, the indented form of the KA has not been conclusively proven to be heritable, but evidence suggests that it likely is.

The beaded presentation is relatively rare and believed to be heritable. Unlike the indented form, there is usually no flattening of the apex. The acrosome has become enlarged and stretched and then folded back on itself over the head of the sperm. Eosin stain does not penetrate the folded, thickened acrosome, resulting in a very distinctive semicircular white structure covering the apex of the sperm head. Typical beaded KA spermiograms contain high percentages of affected sperm and few to no other defects.

KAs seem to have variable, almost confusing effects on fertility. When considering the impact of a high percentage of KAs on fertility it is helpful to consider the two forms. Bulls producing the beaded form are extremely subfertile. Although there were normal-appearing sperm in their ejaculates, it appears that the beaded KAs were not compensable, or that there was an undetected abnormality affecting even the normal sperm. Results of breeding trials using bulls producing high numbers of indented KAs have been variable. In single sire mating situations and artificial insemination studies little to no differences in pregnancy rates were recorded; however, when similarly affected bulls were used in competitive mating trials the KA bulls sired significantly fewer calves. These results implied that normal appearing sperm coexisting with the KA sperm were not as capable of fertilization when competing with normal sperm produced by unaffected bulls [34].

Midpiece Defects

Detached Heads

Detached heads, also termed *free heads or loose heads*, are commonly found in low numbers in semen samples collected during late winter and early spring, suggesting that their occurrence is due to senescence. In these cases, the sperm take up the eosin stain uniformly, indicating that they were dead before ejaculation. Detached heads, both normal and abnormal forms, are also found in variable numbers with other defects following an insult to spermatogenesis. Large numbers of detached normal heads may be seen in cases of sperm accumulation associated with a failure to void aging sperm in the urine. Detached heads may occasionally be encountered that stain alive, usually in the presence of a variety of other defects, indicating an insult to spermatogenesis that has affected the formation of the connection between the head and the midpiece. Detached heads are not a normal phenomenon. Although a few are acceptable they should always be included in the differential count. The one notable exception is when assessing the true proportion of knobbed acrosomes (KAs) in a sample. Dead sperm eventually lose the acrosome; therefore acrosomal anomalies will not be visible. Disregarding dead, detached heads in this case will give a more accurate proportion of KAs present in the spermiogram. (Editor's note: a normal detached or free head may be classified as a midpiece defect because it results from separation at the junction of the midpiece and head.)

Proximal Droplets

Proximal (cytoplasmic) droplets appear in stained preparations as dense white circular structures obscuring the proximal midpiece (Figure 9.15). During the epididymal phase of sperm maturation proximal droplets normally migrate to

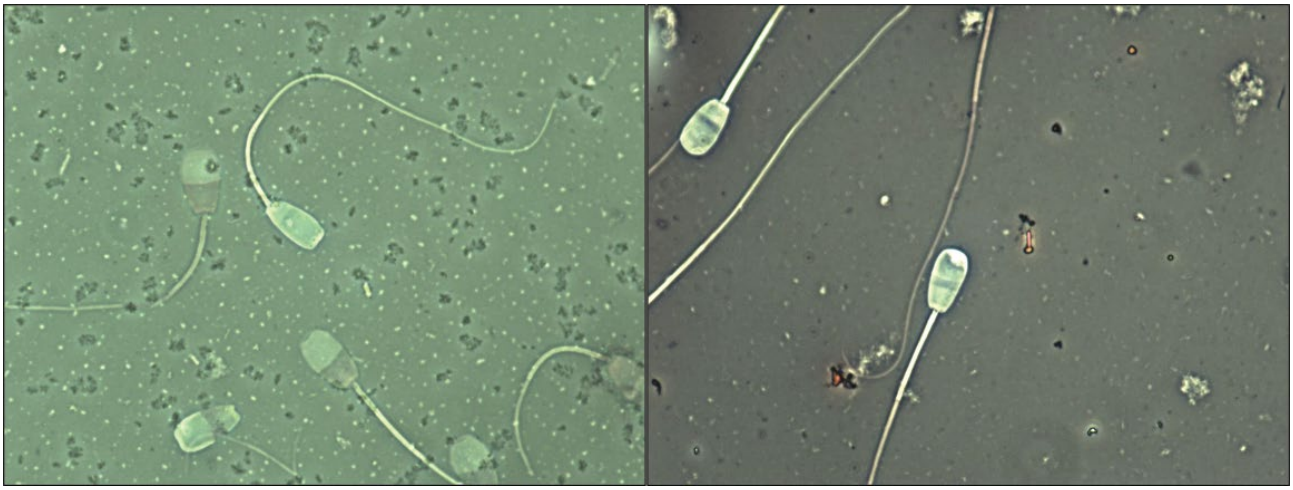


Figure 9.14 Sperm with the knobbed acrosome defect: indented form (left); beaded form (right).



Figure 9.15 Proximal cytoplasmic droplets.

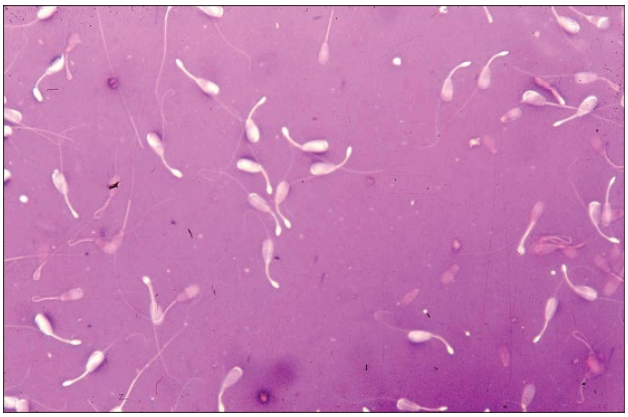


Figure 9.16 Distal midpiece reflexes with and without droplet material and detached heads on an eosin-nigrosin stained smear.

the distal position and are then shed at or around the time of ejaculation. Presence of proximal droplets in a semen smear is indicative of a disturbance of spermatogenesis at the epididymal or testicular level. Within approximately one week of an insult to spermatogenesis, proximal droplets appear in the ejaculate. The short, less than 10-day, time to first appearance of proximal droplets suggests an epididymal origin; however, the reoccurrence of proximal droplets three or four weeks after an insult to spermatogenesis and the well-documented association of high numbers of proximal droplets with sexual immaturity in bulls suggest the involvement of abnormal spermatogenesis at the testicular level as well. Sperm motility is unrelated to the presence of proximal droplets, yet it has been shown that affected sperm lack the ability to bind to ova. This inability to induce a zona reaction would suggest that normal-appearing sperm should be able to compensate for those with droplets, but that is not the case. Normal-appearing sperm produced at the same time as high numbers of sperm with proximal droplets have been shown to have an impaired ability to bind to and penetrate ova. Breeding studies utilizing bulls expressing high numbers of proximal droplets have reported poor conception rates. Proximal cytoplasmic droplets are a serious sperm defect with a profound negative effect on fertility. Clinicians must resist the temptation to give a satisfactory classification to a young bull with a high percentage of proximal droplets in his spermiogram because the amount of time required to clear the defect varies between bulls.

Distal Midpiece Reflexes

Occurring in all breeds of cattle, the DMR is the most common midpiece defect (Figures 9.16 and 9.17). The classic appearance is that of a "J" hook involving the very distal end of the midpiece causing the rest of the tail to be whipped around 180°. Cytoplasmic droplet material is usually trapped in the loop but not always; in the absence of droplet material, examiners should look for the classic "J" hook. Other manifestations include a less-severe 90° bend in the midpiece and second bends involving the principal piece that cause the midpiece and principal piece to loop in a

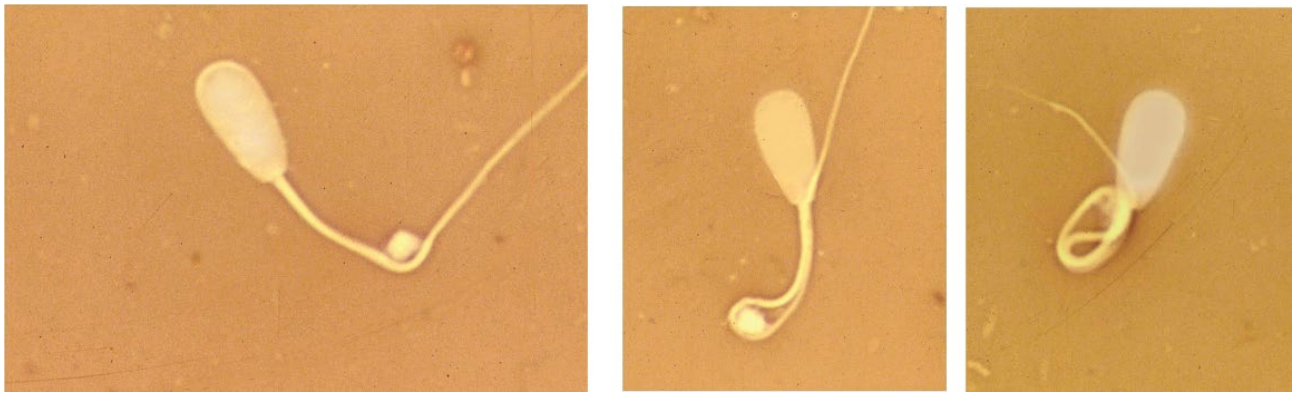


Figure 9.17 Three images all showing distal midpiece reflexes.

circle. The DMR defect occurs in the epididymis associated with a hormonal chain reaction which ultimately causes a decline in testosterone levels. Sperm are stored in the cauda epididymis prior to ejaculation and epididymal sperm possess a distal cytoplasmic droplet which gets trapped in the reflex and is unable to be shed. Both heat and stress may cause a decline in testosterone and the appearance of DMRs. The DMR defect is most often associated with stress insults to spermatogenesis, appearing within just a few days of a stress occurrence. Common stressful events include severe weather, injuries, and illness of at least three or four days duration. With more severe or long-standing events, other defects will appear in succession following the DMR according to where they occur during spermatogenesis. In cold, northern regions DMRs are frequently recorded in semen samples collected during the late winter and early spring, suggesting that photoperiod and/or nutrition may be a cause. This defect is seen in all breeds; however, some animals within a breed appear to be more affected than others by stressful events experienced by the group. When a bull fails to meet the minimum morphology standards for a satisfactory classification due at least in part to a high proportion of DMRs it is appropriate to reevaluate the bull in as little as two weeks. Not only do DMRs appear fast following an insult, but also if all goes well the numbers of affected sperm will decline rapidly.

Mitochondrial Sheath Defects

Surrounding the inner core of the midpiece the mitochondrial sheath is laid down during spermatogenesis, beginning at the attachment of the midpiece to the sperm head all the way to completion at the annulus on the very distal end of the midpiece. Any disruptions in mitochondrial sheath development can result in one or a few tiny gaps appearing along the length of the midpiece (Figure 9.18). Incomplete development of the sheath at the annulus causes the appearance of larger gaps and a propensity for the tail to break at this point. Mitochondrial sheath defects are usually seen in low numbers, but there

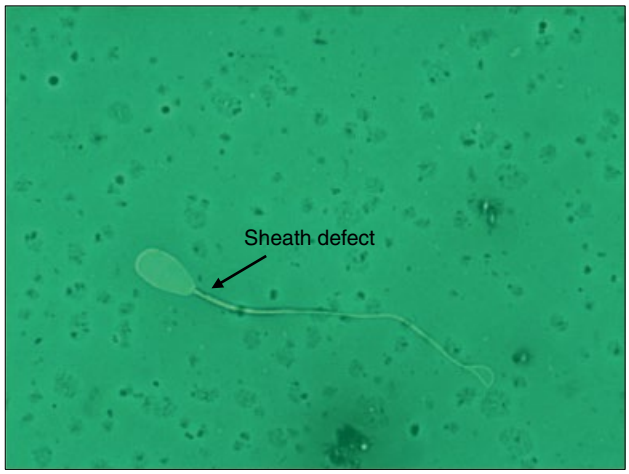


Figure 9.18 Mitochondrial sheath defect.

have been a few bulls that produced a high percentage of sperm with sheath gaps. Surprisingly, these bulls had normal fertility [2]. A conclusive link between the feeding of gossypol in cottonseed meal to bulls and the occurrence of high numbers of mitochondrial sheath defects has been shown [2, 17]. Despite reports of bulls producing high numbers of affected sperm with apparently normal fertility, mitochondrial sheath defects are considered abnormal; therefore any bulls producing high numbers of these defects should be flagged for further examination, including an investigation of possible causes. The more common occurrence is that of a few mitochondrial sheath gaps being noted in spermiograms – usually in the presence of other sperm defects.

Accessory Tails

Accessory tails are an uncommon occurrence affecting a small number to as much as 35% of a spermiogram. Closely related to the abaxial tail aberration, accessory tails, multiple tails, and abaxial tails are often present in the same spermiogram [2]. Differing from abaxial tails, however, accessory tails have been shown to have a negative effect

on fertility. Accessory tails may be partially obscured by droplet material; therefore the proportion of affected sperm may be underestimated. This defect has been recorded in a number of breeds of cattle and is believed to have a heritable basis [27].

Stump Tail

The stump tail defect (Figure 9.19) is unmistakable, with usually more than 50% of the spermiogram affected. Other sperm will have broken midpieces and midpiece defects. Cytoplasmic droplet material often obscures the stump tail from view. This is believed to be a heritable defect reported in Charolais, Hereford, and other breeds of cattle [2].

Dag Defect

The Dag defect is a rare heritable midpiece defect that carries the name of a Jersey bull who produced it in large numbers along with his full brother. The unusual and distinctive name of this defect is often highlighted in the minds of students, leading to a tendency to refer to DMRs as the Dag defect. Differing from the DMR, unique features of the Dag defect include a swollen, disrupted mitochondrial sheath and fractures of the midpiece (Figure 9.20). Oftentimes, midpiece fractures result in coiling of the tail very similar to the DMR. By studying the fractures closely, broken axonemal fibers that can be likened to broken structural cables can be seen. In addition to the Jersey breed, the Dag defect has been reported in the Hereford, Swedish Red and White, and Galloway breeds [2]. Sperm showing the true Dag defect are usually produced in very high numbers. Although very uncommon, a few sperm displaying mitochondrial disruption identical to the Dag defect may be noted in spermiograms, but in the presence of other defects. These defective sperm are called Dag-like to differentiate their occurrence from the rare, heritable condition where high numbers of sperm express the defect in the absence of any other evidence of an insult to spermatogenesis.

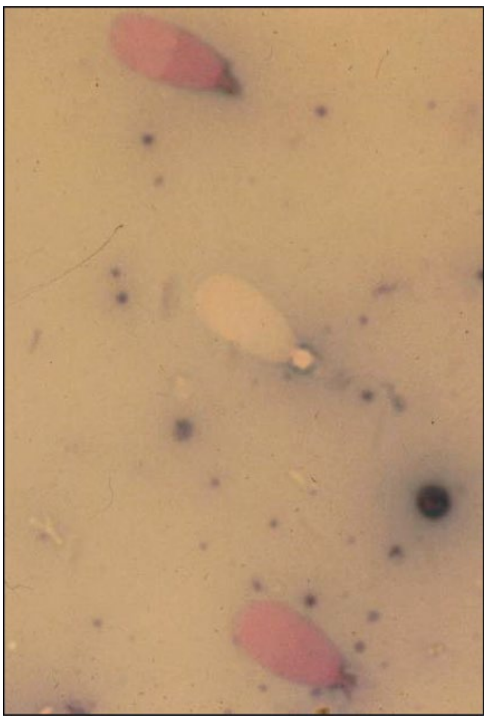


Figure 9.19 Stump tail defect.



Figure 9.20 Dag defect.

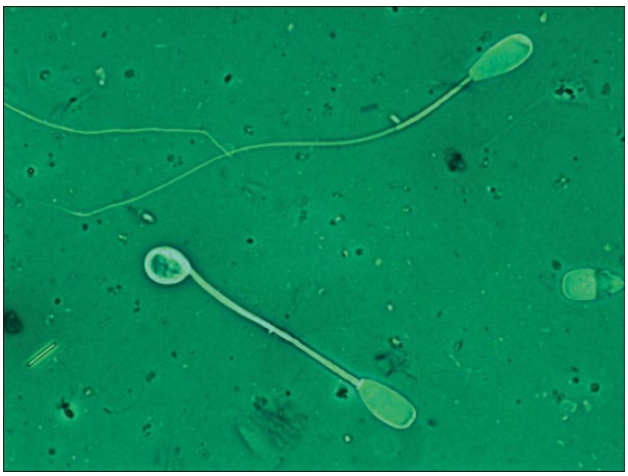


Figure 9.21 Coiled principal piece.

Principal Piece Defects

Coiled Principal Piece

Coiled principal pieces can be found in small numbers on many smears, often with other defects (Figure 9.21). They usually appear at the same time as nuclear vacuoles following an insult to spermatogenesis. Sperm with coiled principal pieces are not capable of normal motility.

Bent Principal Piece

Occasionally, sperm displaying a loop bend in the principal piece with cytoplasmic droplet material trapped in the loop are encountered. The loop begins just past the midpiece, so technically it is not a DMR. Most of the sperm with bent principal pieces are noted in spermiograms where DMRs are present and are believed to have the same origin.

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10

Ultrasound Examination of the Reproductive Tract

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Introduction

Diagnostic ultrasound has become the standard for medical imaging of soft tissue. The genital tract is an obvious target for this diagnostic modality and while ultrasound is routinely employed for evaluation of the female genital system its use in male domestic animals remains much less common. In the bull, for example, the routine bull breeding soundness examination (BBSE) as recommended by the Society for Theriogenology does not require ultrasonographic assessment of the genital system [1, 2]. In many cases of infertility in the bull, adequate diagnostic and prognostic information is obtained without the use of ultrasonography. A major advantage of ultrasonography is the ability to localize and objectively assess morphologic changes in tissue.

A thorough history, complete physical examination, and a BBSE should accompany an ultrasound examination of the bull's genital system. We routinely perform ultrasound evaluations for bulls with palpable or visible abnormalities of the genital system; those with pyospermia, hemospermia, azoospermia, or oligospermia; those with low semen volume; and bulls that persistently produce high numbers of morphologically abnormal or immotile sperm. As with any medical technology, the use of ultrasonography for examination of the bull will expand as veterinarians become more familiar with the modality and its applications.

Equipment and General Methodology

A wide variety of ultrasound equipment is available for diagnostic imaging. Most veterinary clinics providing service to cattle owners will already have a portable, real-time, B-mode system with a linear intrarectal probe that is used for reproductive examination of cows. All illustrations in this chapter were obtained with this type of system. Typical probe frequencies range from 5 to 7.5 MHz and are more than adequate for imaging the bull's reproductive tract. More detailed images of the epididymis and penile tissues can be obtained using probes with a smaller footprint and higher frequency. Representative images may be captured, labeled, measured, and saved for inclusion in the medical record. These applications are available with most ultrasound systems.

In order to adequately assess a machine that you intend to purchase, it is advisable to use it in an actual clinical setting so you can critically evaluate functionality and image quality under real-world conditions. Beyond the primary issue of image quality, durability and resistance to damage from dirt and moisture are important considerations when purchasing an ultrasound unit to be used in cattle environments.

Operator safety is always a concern when working with bulls. A chute or stock with a head catch and squeeze option is ideal. For added security, the bull's head can be secured with a halter. Most bulls will tolerate scrotal, perineal, and rectal examinations with little restraint beyond confinement in the chute. If a kick bar is used behind the bull, extreme caution is needed to avoid placing the arms and hands in a dangerous position. For fractious bulls or for examination of the prepuce and penis cranial to the scrotum, we prefer sedation (xylazine 0.01–0.02 mg/kg IV).

While it is beyond the scope of this chapter, a fundamental understanding of ultrasound principles and an appreciation for common image artifacts are essential for anyone offering diagnostic ultrasonography services. For those requiring a more expansive treatment of the basic principles of veterinary ultrasonography, excellent texts are

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

available [3, 4]. A bovine reproductive anatomy monograph is available from the National Association of Animal Breeders [5] and McEntee [6] is an excellent resource for those seeking a review of the pathology of the male genital system.

Images are obtained by either a transcutaneous or transrectal scan depending on the anatomical location of the tissue to be examined. If long or dense hair covers the area, the quality of the transcutaneous image is improved by first shaving the skin. Shaving is usually not required for examination of the scrotum and testes. Image quality is enhanced when the scrotal skin is stretched tightly over the testes. To do this, firmly grasp the scrotal neck dorsal to the testes and pull the testes ventrally while conducting the examination.

In all cases of transcutaneous ultrasonography, a coupling agent is required to achieve good contact between the probe and skin. Any air or gas between the probe and the tissue to be examined will interfere with the acquisition of an image. The use of ultrasonic coupling gel as a coupling agent has the advantage of long duration, since it does not evaporate. Additionally, it is approved by most manufacturers for contact with the probe surface. Alcohol (70% isopropyl) also makes a good coupling agent, with the advantage that it does not have to be cleaned from the probe or patient after the examination is completed. A disadvantage of alcohol is that it evaporates and often needs to be reapplied to complete the examination. Additionally, not all probe surfaces are approved for contact with alcohol or other solvents. Placing a protective cover containing a small amount of coupling gel over the probe will prevent alcohol from contacting the probe surface. A disposable examination glove works well for this purpose.

For transrectal examination of the pelvic organs, the probe is covered with an examination sleeve containing just enough coupling gel to provide good contact between the probe and sleeve. Most probes designed for transrectal use in horses and cattle will fit snugly within a finger of a glove or sleeve. The feces are evacuated from the rectum and a manual examination is conducted before the covered probe is inserted. No coupling agent is necessary to maintain contact between the sleeve and rectal mucosa beyond the lubricant normally used to facilitate insertion of the probe and arm. If a protective cover is not used, the probe should be cleaned and disinfected between examinations following the recommendations of the probe manufacturer.

While diagnostic ultrasonography is generally regarded as safe for examination of reproductive tissues and fetuses [4], few published data exist in the veterinary literature. Coulter and Bailey [7] exposed yearling beef bulls to ultrasound (three minutes for each testis, one time, using a 5-MHz linear transducer) and found no discernible effects on sperm numbers, morphology, or motility during a 70-day period after the examination. They concluded that diagnostic ultrasonography should be safe for examination of the bull scrotum and testes. A reasonable approach is to use the minimum power and contact time necessary to complete your examination.

Examination of the Scrotum

B-mode ultrasonography of bull testes was first described by Pechman and Eilts [8] in 1987. A thorough visual and manual examination of the scrotum and its contents should always precede the ultrasound examination. The scrotum and testes are then examined by ultrasound in both a sagittal and transverse plane, with the probe applied to the cranial, lateral, or caudal surface of the testis depending on the examiner's preference. A complete examination includes the spermatic cord, the entirety of the testis parenchyma, the epididymis, and the scrotal wall. The body of the epididymis and the ductus deferens are difficult to detect unless they are grossly abnormal.

Normal ultrasonic appearance of the testis is shown in Figure 10.1. The testis parenchyma appears as a homogene-

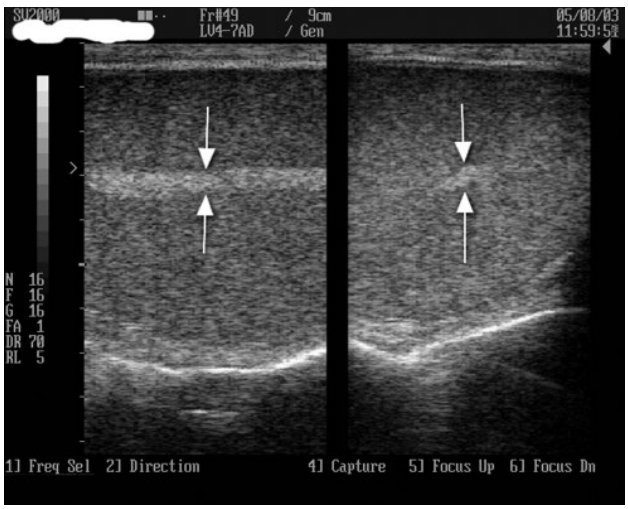


Figure 10.1 Caudal ultrasonographic views of the left testis of a yearling dairy bull. Caudal is at the top and cranial at the bottom of each ultrasound image. On the left is a sagittal view, with the mediastinum testis (between arrows in each image) appearing as a more hyperechoic linear structure running through the approximate center of the less echoic testis parenchyma. On the right side of the image is a transverse view of the same testis, with the mediastinum in cross-section, again in the middle of the less echoic parenchyma. The scrotal wall is visible adjacent to the probe on the top (caudal) and on the bottom (cranial) of the testis in each image. The bright white line at the bottom of each image is a gas artifact that marks the far side of the scrotal skin.

ous tissue with a stippled medium echogenicity that surrounds the centrally located and more hyperechoic (whiter) mediastinum testis. The mediastinum testis is normally less than 5–6 mm wide. The parenchyma consists primarily of convoluted seminiferous tubules that contain the sustentacular (Sertoli) and germinal cells but also includes the adjacent interstitial (Leydig) cells along with associated vascular, neural, and stromal tissues. The seminiferous tubules are connected by the straight tubules to the rete testis that comprises a series of interconnected channels within the mediastinum testis. Sperm pass dorsally through the rete testis to the efferent ducts that in turn penetrate the capsule of the testis before uniting as the epididymal duct.

Changes in the ultrasonic appearance of the testis parenchyma can reflect normal events around puberty as well as pathology. An increase in echogenicity (increased pixel intensity, or a whiter parenchyma) has been reported to occur in association with sexual development around the time of puberty [9, 10], but ultrasonography was no better at predicting puberty than was measurement of scrotal circumference [10]. While pixel intensity has been correlated with attainment of sexual maturity as assessed by semen quality [11], it seems to be a better predictor of future semen quality than the present status of the bull [12]. Subsequent studies in mature bulls have failed to correlate pixel intensity with semen quality at BBSE [13] or with histologic measures of spermatogenesis [14].

In any event, objective assessment of these subtle changes in pixel intensity is not possible by real-time visual interpretation of an ultrasound image. To accurately assess pixel intensity, images must be obtained and digitally preserved using rigorously standardized methods. The digital files are then sampled and evaluated using computer software that can objectively assess the gray scale of each pixel in the sampled area, providing both an average intensity and measure of variation. Given the limited clinical utility and the great effort required, Brito et al. [10] concluded that "though testicular ultrasonogram pixel intensity might be useful for research purposes, clinical application of this technology in the present form for bull breeding soundness evaluation is not justifiable."

The majority of the discrete anomalous ultrasonographic changes detected in the testis are more hyperechoic than normal tissue. These may appear as small foci scattered throughout the testis (Figure 10.2) or they may be more localized (Figure 10.3). Larger hyperechoic lesions are often seen to radiate from the rete to the periphery of the testis, suggesting the complete involvement of one or more seminiferous tubules (Figure 10.4). The histologic basis for the increase in echogenicity is usually fibrosis [15] but the echogenicity itself does not confirm histology or etiology. Where a specific histologic diagnosis is provided in the accompanying ultrasound images, it is based on histopathologic assessment of excised tissues.



Figure 10.2 Sagittal view of the left testis of a mature bull with small hyperechoic foci distributed throughout the testis parenchyma.

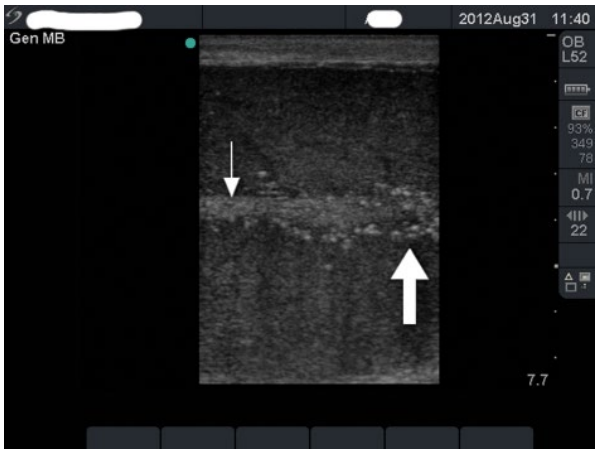


Figure 10.3 Sagittal view of a testis with small hyperechoic foci (large arrow) surrounding the mediastinum (small arrow) suggestive of lesions in the straight ducts that drain the seminiferous tubules into the rete testis.



Figure 10.4 Sagittal view of a testis with multiple linear radiating hyperechoic lesions, two of which are highlighted by the smaller arrows. A normal mediastinum testis is shown (large arrow).

Tumors are generally more hyperechoic than the normal testis parenchyma (Figure 10.5), but mixed or hypoechoic echogenicity is also possible [4]. Mineralized lesions are extremely hyperechoic and are usually accompanied by intense or complete shadowing causing a loss of image distal to the lesion (Figure 10.6).

Barth et al. [15] have proposed a scoring system for fibrotic lesions in the bull testis based on the size and frequency of hyperechoic lesions detected by ultrasonography in a single transmediastinal plane. They found that the occurrence of fibrotic lesions in young bulls aged between 3 and 20 months was not associated with decreased semen quality. This is consistent with their histologic findings, where seminiferous tubules within the area of fibrosis were generally affected, but immediately adjacent germinal tissue was quite often completely normal. However, bulls that experienced a dramatic increase in fibrosis score at a second examination (≥4 points, 4–13 months later) were more likely to have poor semen quality than bulls that did not experience this degree of change [15]. While bulls in this report with up to 50% of their parenchyma affected were able to produce mostly normal sperm, it is reasonable to suspect that they would have reduced production capacity.

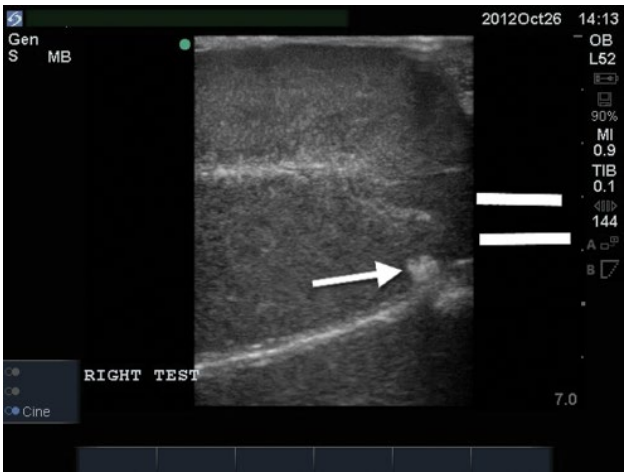


Figure 10.5 Sagittal view of the right ventral testis of an aged beef bull. Histologically, the hyperechoic lesion (arrow) was a Sertoli cell tumor.

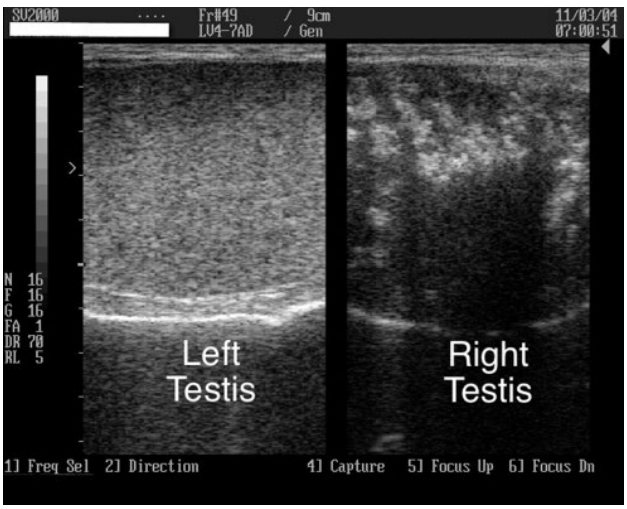


Figure 10.6 Sagittal views of the left and right testes of a yearling dairy bull. The left testis is normal, while the hyperechoic areas of the right testis cast prominent dark shadows in the distal tissue and represent areas of mineralization.

The cause of fibrotic lesions in the testis is unknown. Possible etiologies include infectious or inflammatory conditions, developmental defects of the seminiferous tubules or their connecting ducts, autoimmunity, degenerative conditions, or aging. Ram testes inoculated with *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) eventually developed hyperechoic lesions that consisted of fibrotic tissue [16]. A severe outbreak of bovine respiratory syncytial virus (BRSV) in a group of bulls was associated with an increased prevalence of fibrotic lesions in one report, though a cause-and-effect relationship could not be confirmed [15]. On the other hand, scrotal insulation resulting in a dramatic decrease in semen quality did not cause fibrosis or any other ultrasonically detectable change in the testis within four months [17] or six months [12] after the insulation event. A progressive fibrosis that is more marked in the ventral testis has been described as a histologic feature of aging in the bull [18].

Distinct hypoechoic lesions in the testis parenchyma are seen less commonly (Figure 10.7). Intratesticular inoculation of *T. pyogenes* in rams caused an initial hypoechoic change associated with edema, neutrophilic infiltration, and degenerative changes in the seminiferous tubules [16]. Tumors may present as hypoechoic areas in some cases. Since an increase in echogenicity is seen at puberty in association with proliferation of the spermatogenic tissue, it seems reasonable to speculate that areas affected by a degenerative process would be more hypoechoic by contrast.

A hypoechoic distension of the rete testis was first reported in Ayrshire cattle [19]. Most of the bulls in this report had bilateral distension and were azoospermic by 16 months of age, suggesting a block to sperm outflow. A genetic predisposition to the condition was suspected. A similar unilateral lesion was reported in a mature Holstein bull in conjunction with agenesis of the ipsilateral seminal vesicle, epididymal body and tail, as well as low semen volume [20]. Figure 10.8 shows an ultrasonogram from a yearling Holstein bull with a history of oligospermia and low semen volume. Note the enlarged hypoechoic appearance of the mediastinum. The contralateral testis and mediastinum appeared normal (not shown). Surgical removal of the

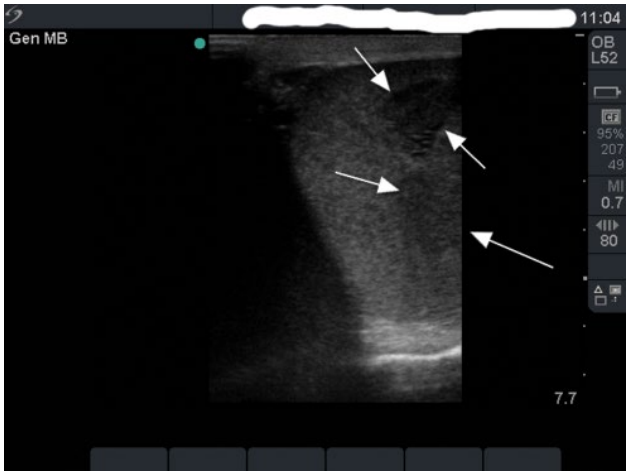


Figure 10.7 Transverse view of the testis containing two discrete hypoechoic wedge-shaped areas (between arrows) of unknown etiology or significance.

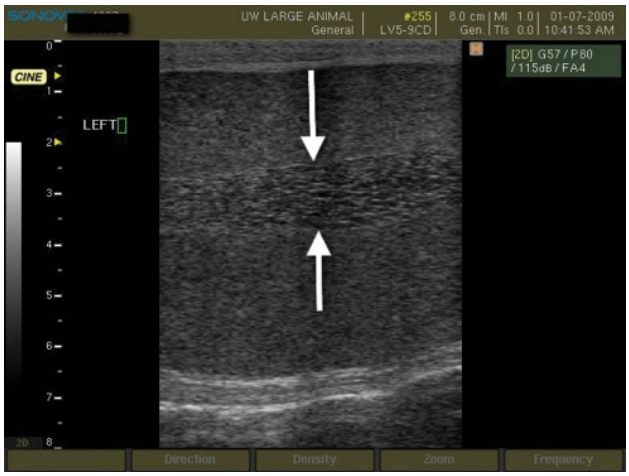


Figure 10.8 Sagittal view of the left testis of a mature Holstein bull that presented with a complaint of low semen volume and oligospermia. Note the hypoechoic distension of the rete testis (dark linear area between arrows). The distension was secondary to a complete outflow obstruction resulting from the absence of the epididymal body on this side.

affected testis confirmed a diagnosis of agenesis of the body of the epididymis and fluid distension of the rete testis. The distension can also affect the efferent ducts. In our experience, extensive and uniform hypoechoic distension of the rete testis as seen in Figure 10.8 is pathognomonic for sperm outflow obstruction, most often congenital. In unilateral cases, the presenting complaint is usually low sperm numbers and semen volume in an otherwise normal young bull. Congenital cases may be associated with aplasia affecting the epididymis, deferent duct, ampulla, or seminal vesicle.

The head of the epididymis is best seen in a sagittal view and is found on the dorsocranial aspect of the testis. It is usually less echoic than the parenchyma of the testis. The

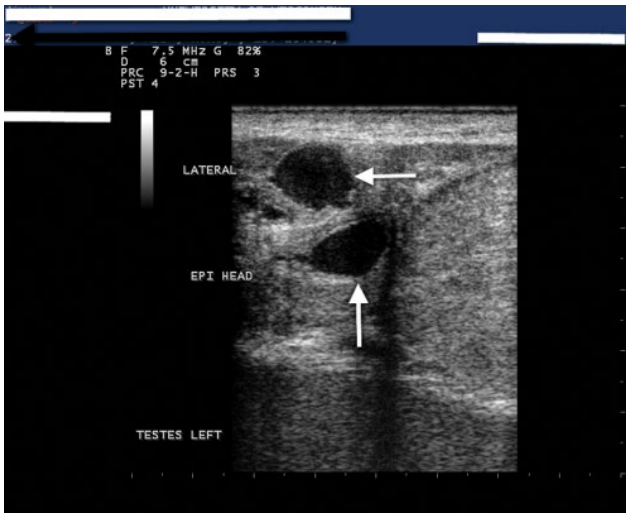


Figure 10.9 Sagittal view of the testis. Note the hypoechoic dilations (arrows) in or near the head of the epididymis. Normal testis parenchyma is seen to the right of the cysts.

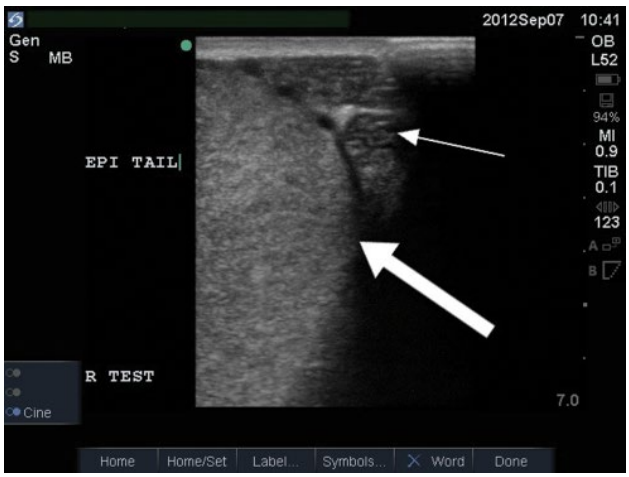


Figure 10.10 Sagittal view of the ventral testis (large arrow) and tail of the epididymis (small arrow) of a normal bull.

tail of the epididymis can be readily located by palpation and is found on the ventral aspect of the testis. Acquired lesions of the epididymides can lead to outflow obstruction. A cystic distension of the head of the epididymis is shown in Figure 10.9. It is worth noting that cysts of the appendix epididymis (proximal mesonephric duct remnant) are also found in this area and even those as large as a few centimeters in diameter usually do not interfere with normal epididymal function [6]. The epididymal tail (Figure 10.10) can be difficult to image with a transrectal probe and for fine detail one should consider using a higher-frequency probe with a smaller footprint. Congenital, traumatic, and inflammatory lesions can be seen here, with sperm stasis and granuloma formation as possible sequelae. Alterations

of size and echogenicity are characteristic of disease in this tissue. Multiple small sperm granulomas are visible as focal hyperechoic lesions in the tail of the epididymis (Figure 10.11).

Ultrasonography can provide more specific information about the extent, location, and resolution of scrotal pathology. Examples include scrotal hydrocele (Figure 10.12), inflammation, sperm granuloma, spermatocele, fibrosis, mineralization, herniation, tumors, abscesses, trauma, hemorrhage, outflow obstruction, and lesions of the scrotal wall and vasculature. The echogenic pattern seen is variable and often quite heterogeneous. In cases of testicular trauma (Figure 10.12), ultrasound can confirm the actual degree of damage to the testis and provide a more accurate prognosis for recovery. Ultrasound can be used in cases of scrotal hernia to confirm the presence of herniated bowel as well its viability as assessed by the presence or absence of peristalsis.

The final element of the scrotal ultrasound evaluation is an assessment of the scrotal neck and spermatic cord (Figure 10.13). While herniation, trauma, hemorrhage, and scrotal hydrocele can affect this region, they are usually more evident in the scrotum proper. The vas deferens cannot be reliably imaged in normal bulls. The primary ultrasonic feature of this region is the vascular cone consisting of the prominent tortuous testicular artery overlain by a fine network of very small veins called the pampiniform plexus. Brito et al. [21] reported an increase in vascular cone diameter up to 13.5 months of age in beef bulls and increased vascular cone diameter was positively correlated with an increased percentage of normal sperm.

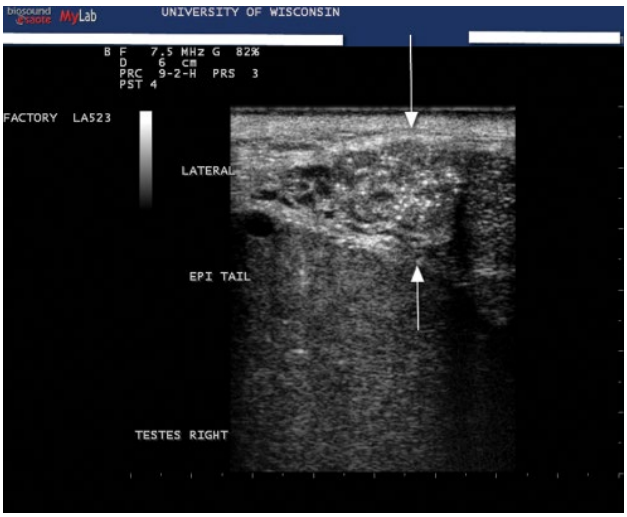


Figure 10.11 Transverse view of the ventral aspect of the right testis of an aged beef bull. The tail of the epididymis is seen between the arrows. The small white foci seen in the epididymal tissue between the arrows are sperm granulomas.

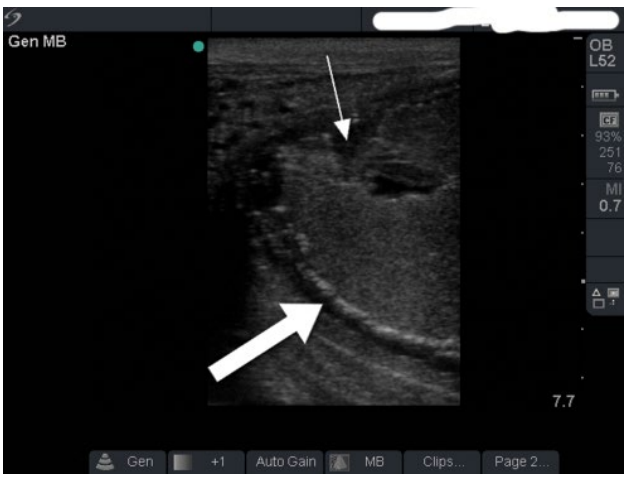


Figure 10.12 Sagittal view of the dorsal testis. The small arrow points to a traumatic lesion of the testis capsule and visceral vaginal tunic. The hypoechoic fluid surrounding the testicle is a persistent scrotal hydrocele (large arrow).

Examination of the Pelvic Organs

The anatomy of the pelvic accessory sex organs is reviewed in Chapter 1. Ultrasonic assessment of the accessory glands of the bull was first described in 1987 by Weber et al. [22] Transrectal ultrasound images are generally sagittal but may also be oblique to the long axis. An ultrasonic evaluation of the tissues is performed after first evacuating fecal material and manually palpating the reproductive tract. The transverse band of prostatic tissue on the dorsal aspect of the cranial extent of the pelvic urethra is a convenient landmark (Figure 10.14). The pelvic urethra is then imaged in a caudal direction (Figure 10.15) until the paired

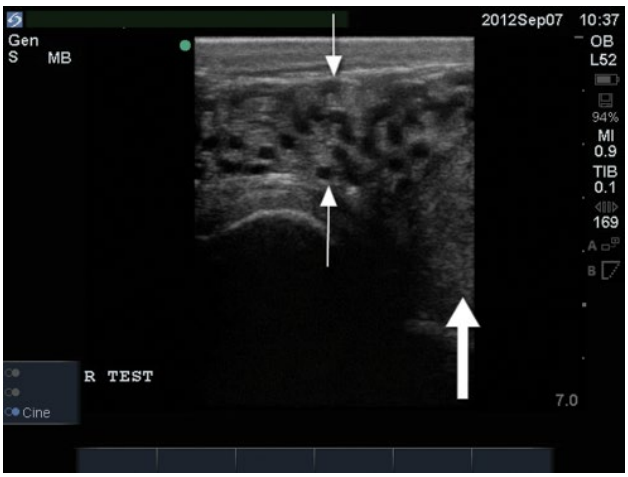


Figure 10.13 Sagittal view of the vascular cone (between small arrows) just dorsal to the testis (large arrow). The lumen of the testicular artery is represented by the black irregular areas in the vascular cone. The network of veins forming the pampiniform plexus is not usually visible due to their small size.

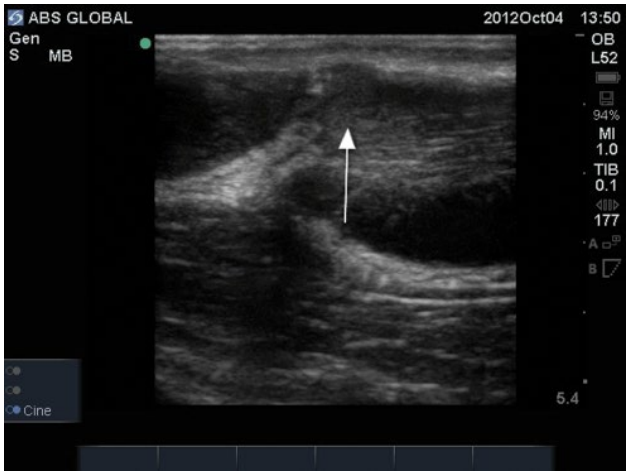


Figure 10.14 Midline sagittal view of the body of the prostate gland (ovoid tissue above arrow). Dorsal is on top, ventral on the bottom, cranial to the left, and caudal to the right.

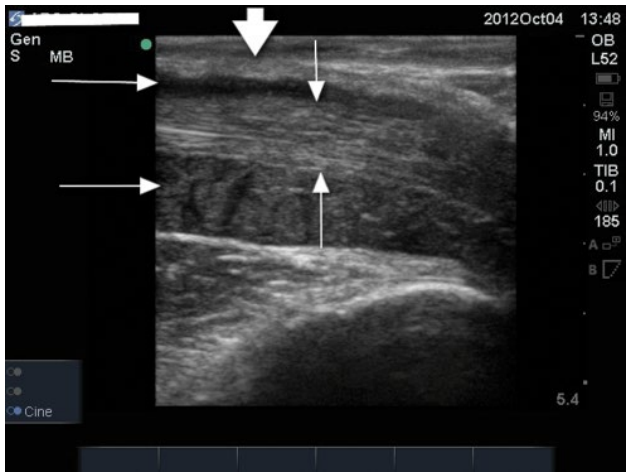


Figure 10.15 Sagittal view of the urethralis just caudal to the body of the prostate. The horizontal arrows on the left point to the more hypoechoic urethral muscle above and below the urethra. The more hyperechoic tissue between the vertical arrows is the cavernous tissue immediately surrounding the pelvic urethra. The disseminated prostate is seen as a hyperechoic linear structure on the dorsum of the urethralis (large vertical arrow). Dorsal is on top, ventral on the bottom, cranial to the left, and caudal to the right.

bulbourethral glands are detected (Figure 10.16). Moving cranially, the urethralis is followed until the prostate is encountered again. The paired ampullae are found just cranial to the prostate, on or near the midline (Figure 10.17). The paired vesicular glands are found just lateral to the ampullae (Figure 10.18). An ultrasonic assessment of the bladder should also be done at this time to check for anomalies, cystitis, or calculi.

Pathology of the bulbourethral glands and prostate is uncommon. Aplasia [6] and sperm accumulation [23] have

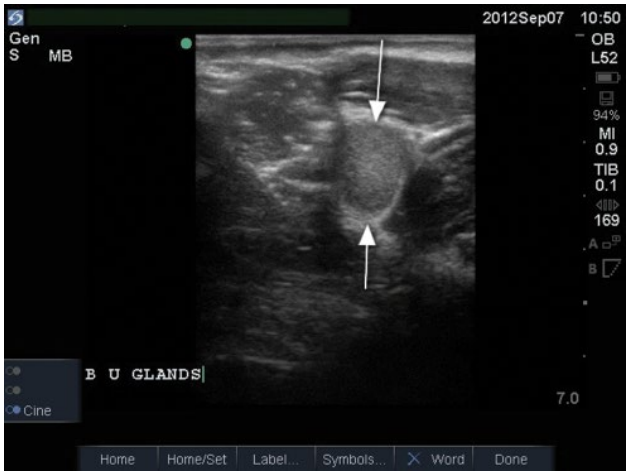


Figure 10.16 Sagittal view of the bulbourethral (Cowper's) gland (between arrows). Dorsal is on top, ventral on the bottom, cranial to the left, and caudal to the right.

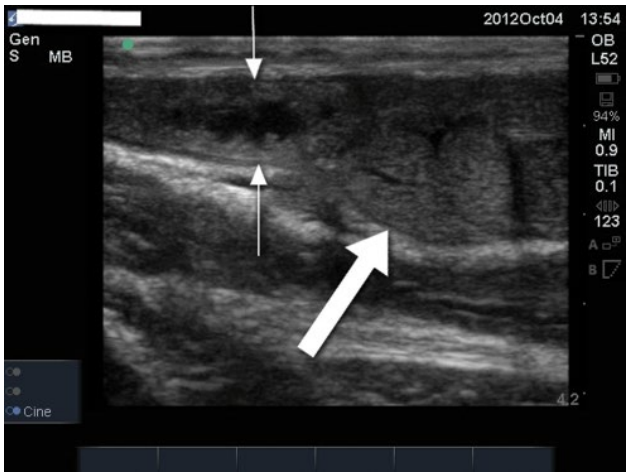


Figure 10.17 Sagittal view of the ampulla (between small arrows). Note the distended hypoechoic lumen surrounded by gland tissue. Luminal contents may or may not be seen during examination of normal bulls. The lobar tissue above and to the right of the large arrow is an adjacent seminal vesicle. Dorsal is on top, ventral on the bottom, cranial to the left, and caudal to the right.

been reported in the ampullae. Inflammation of the vesicular glands is relatively common in bulls and should be suspected whenever an asymmetry in size or echotexture is encountered. Inflamed glands are larger and often have a mixed echogenicity, while abscessed glands show a loss of lobar architecture and often have a globoid area of variable echogenicity [24]. A bacterial infection is most commonly incriminated as the cause of seminal vesiculitis, but sterile inflammation resulting from urine or sperm leaking into the glands is also a potential etiology. Figure 10.19 is an ultrasonogram from a two-year-old beef bull with segmental aplasia of the right vesicular gland. Note the cystic dilation



Figure 10.18 Oblique view of the seminal vesicle (horizontal tissue between arrows). Note the lobar architecture. A short segment of the hypoechoic central duct is seen in the center of the gland between the two arrows.

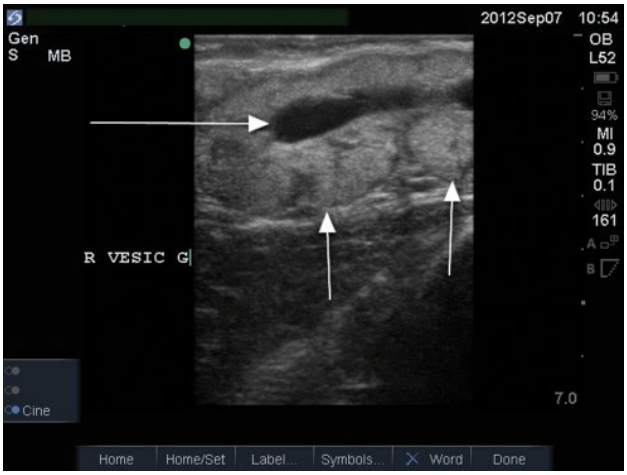


Figure 10.19 Oblique view of the seminal vesicle (above vertical arrows). This bull had a segmental aplasia of the seminal vesicle on this side resulting in the cystic distension of the central duct (horizontal arrow).

of the occluded central duct. The gland was much larger compared with the gland on the left. This bull also had aplasia of the right epididymal tail and a grossly distended hypoechoic right rete testis.

Examination of the Penis and Prepuce

Ultrasound can be used to examine the penis, prepuce, and penile urethra. Ultrasonography of the perineal area caudal and dorsal to the scrotum will generally require shaving the hair to obtain good images. The risks associated with ultrasound examination of the penis and prepuce cranial to the scrotum should be balanced with the lower probability of the examination providing valuable diagnostic and prognostic information beyond that obtained by visual inspection and palpation. Direct observation of the penis and prepuce during mating or semen collection is necessary to detect many conditions, such as penile hypoplasia, genital warts, persistent frenulum, hair rings, or balanoposthitis. Ultrasonically relevant lesions affecting the penis and prepuce include trauma, neoplasia, hematoma, abscess, calculi, urethral dilation and rupture, and vascular shunts.

Alternative Ultrasound Modalities

While B-mode ultrasonography is currently the most commonly employed modality for examination of reproductive tissue, other ultrasound-based diagnostic methods exist. Color Doppler, contrast-enhanced ultrasonography and tissue elastography are adjunct imaging modalities used to investigate lesions in human testes [25] as well as other tissues.

The direction, relative velocity, and volume of blood flow in arteries can be assessed by use of Doppler ultrasonography [4]. A color Doppler ultrasonogram of the testicular artery dorsal to the testis within the vascular cone is shown in Figure 10.20. In a recent report using Doppler ultrasound, the resistive index in both the marginal and intratesticular portions (Figure 10.21) of the testicular artery of the bull were correlated with the total sperm in the ejaculate, immature sperm, abnormal sperm, and presence of the Dag defect [26]. Because of the relative ease of marginal testicular artery imaging (located medial to the testis), the

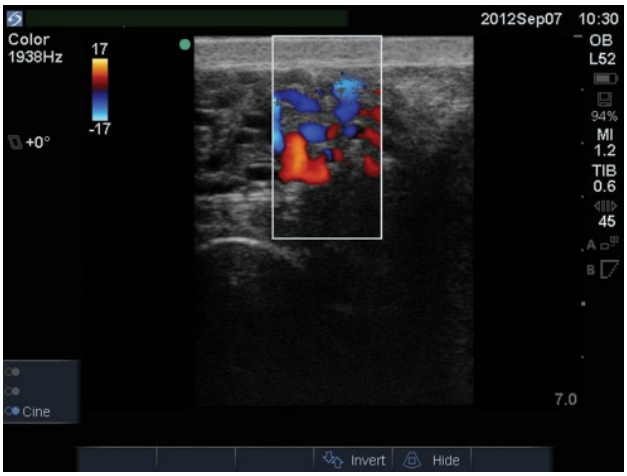


Figure 10.20 Sagittal view of the vascular cone using color Doppler mode. The direction of blood flow in the supratesticular portion of the testicular artery is indicated by color and the velocity by shade within color.

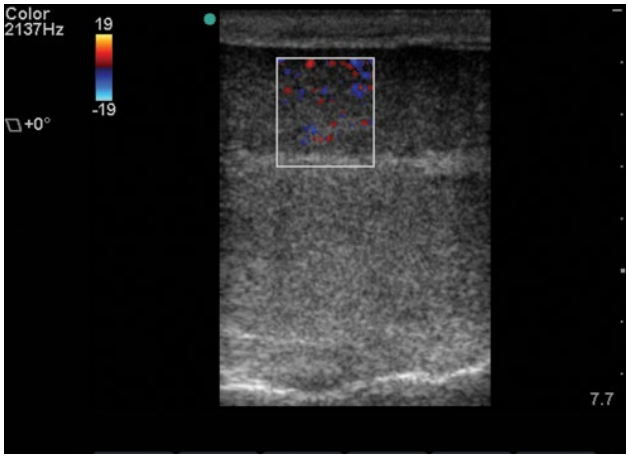


Figure 10.21 Sagittal color Doppler image of the testis illustrating blood flow (colored areas) in the intratesticular portion of the testicular artery.

authors conclude that this could be an "easy-to-perform parameter to evaluate the spermatogenesis quality" in bulls. And while the correlations in this report were significant, there was no evidence that they could be used to establish cut-off values to distinguish between satisfactory and unsatisfactory breeding bulls.

Tissue elastography measures the stiffness of tissue in response to an applied force. This force can be applied either mechanically or by the ultrasound waves themselves. A soft consistency of the testis is present in cases of testicular degeneration [27], but manual assessment of testis consistency is limited by both its subjective nature and the presence of intervening scrotal wall with varied skin texture and fat deposits. Mechanical systems that compress the scrotal surface have been proposed [28], but they still have limitations as they must be applied to the scrotal surface and do not directly measure the consistency of the testis. Tissue elastography offers the advantage of both more objective assessment of tissue stiffness and the ability to focus on the tissue of interest within the testis. Most focal lesions of the testes will differ in consistency in comparison to the surrounding normal parenchyma.

It is important to note that most inexpensive portable bovine ultrasound systems lack a color Doppler function and even much more expensive systems do not routinely offer elastography. Given the cost and limited clinical application in bulls, these modalities will be of more interest to researchers than to clinicians for the foreseeable future.

Summary

Diagnostic ultrasound is a safe, non-invasive method for acquiring additional diagnostic and prognostic information in cases of infertility in the bull. Clinical competence with bull genital ultrasonography can be readily achieved by routinely imaging the genital tract of normal healthy bulls. This will provide the frame of reference necessary to identify and interpret pathologic changes as they are encountered. The common lesions are seldom subtle and are readily detected by systems providing average or better image quality. We recommend ultrasonography as an adjunct to the BBSE for all cases of subfertility in artificial insemination (AI) sires and valuable natural service sires. Routine screening of young sires as part of a pre-purchase evaluation should be done with caution as the most common ultrasound change encountered is a fibrotic lesion or foci in the testes. These lesions have been shown to have little or no effect on semen quality in young bulls. Advances in ultrasound diagnostic capability, especially as it relates to assessment of blood flow in the scrotum or tissue density measurement, may enhance our ability to discriminate between fertile and infertile bulls in the future.

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11

Management of Breeding Bull Batteries

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Introduction

The purchase and maintenance cost of the bull battery represents a significant expense to the cow calf producer. Improper management of this investment can have negative economic consequences through both a reduction in herd productivity and the loss of bulls due to injury or death. A reduction in the size of the calf crop or loss of the calf crop is easily noticed by the producer, but many subfertile bulls are capable of inefficiently producing offspring and this may go unnoticed in some herds. This inefficiency leads to delayed conception, which has been estimated to cost 23–27 kg of weaning weight for every 21 days a cow remains open during the breeding season [1]. In an attempt to compensate for poor bull management, producers will often increase the stocking density of bulls within their herd. The expense of purchasing and maintaining excess bulls can also limit the profitability of a herd.

The management of breeding bulls will vary somewhat between herds, but the overall goal should be to provide a group of disease-free, structurally sound, and physically fit fertile bulls with good libido. Achievement of this goal requires an understanding of both the general and reproductive health requirements of breeding bulls and how these requirements change during the production phase of the bull. The production phase of a breeding bull can be divided into three time periods: the pre-breeding period, breeding period, and non-breeding period.

Pre-Breeding Period

The pre-breeding period begins 60–90 days prior to the start of the breeding season. Breeding soundness evaluations, vaccinations, and diagnostic testing for reproductive pathogens should all occur at the beginning of this period. Performing these procedures early gives the producer time to find replacements if necessary. Vaccination prior to the start of the breeding season should provide protection to the bull while reducing or eliminating his significance as a disease reservoir or vector. Vaccinating bulls at least 60 days prior to the breeding season should allow any viral shedding or transient negative effects on spermatogenesis to subside before breeding. Body condition can also be assessed at this time and minor adjustments to the diet made if necessary.

Breeding Soundness Evaluation

Breeding soundness evaluations should be performed by a veterinarian on all bulls prior to the breeding season. Any bulls deemed unsatisfactory potential breeders, by the standards for breeding soundness of the Society for Theriogenology, should not be used for breeding and bulls placed in the deferred category should be reevaluated prior to use. Herds with two breeding seasons should have their bulls examined before each season. The complete breeding soundness evaluation of breeding bulls is covered in Chapters 7, 8, and 9. Lameness and other foot problems are often first identified during the breeding soundness evaluation. Performing these examinations early will allow for treatment and corrective trimming prior to breeding, possibly salvaging bulls that may have been culled otherwise.

Vaccination

As a general recommendation, breeding bulls should be vaccinated for the same pathogens as the mature cow herd. Vaccination should include protection against clostridial diseases, leptospirosis, campylobacteriosis, bovine viral

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

diarrhea virus (BVDV), and bovine herpesvirus 1. Vaccine efficacy and safety varies between vaccine types and products. The goal of this section is not to endorse specific products but to demonstrate the role vaccination plays in control of these pathogens and to address any negative impacts vaccination may have on bull fertility.

Clostridial Diseases

Clostridial diseases are not transmitted directly between cattle and are not considered reproductive pathogens, but they are invariably fatal. Therefore vaccination is solely aimed at protection of the bull. All clostridial vaccines are killed products and should be boostered according to the label directions. Annual revaccination is recommended especially in all cattle two years of age and younger [2].

Leptospirosis

Leptospirosis is a common cause of abortion and infertility of cattle worldwide. More than 200 serovars of *Leptospira* have been identified, each of which is supported within its own maintenance host. The severity and chronicity of disease caused by organisms of the genus *Leptospira* depends on whether infection is taking place in the maintenance host (host-adapted) for that specific serovar or an incidental host (non-host-adapted) [3]. Cattle are the maintenance host for serovar *hardjo*, which contains two genetically distinct types: *Leptospira interrogans* serovar *hardjo* type *hardjoprajitno* found primarily in the British Isles and *Leptospira borgpetersenii* serovar *hardjo* type *hardjo-bovis* found throughout the world including North America [4, 5]. Infection of cattle with a non-host-adapted serovar is characterized by acute disease, late-term abortion, and transient renal shedding of the organism. Infection with the serovar *hardjo* is characterized by chronic infections, prolonged renal shedding, abortion, and infertility [4]. *Leptospira* species are primarily shed in the urine of infected cattle. Transmission is possible via semen, and venereal transmission of serovar *hardjo* is thought to be common [3, 6].

Vaccination of cattle against leptospirosis is a complex and controversial subject. Many pentavalent vaccines marked for prevention and control of leptospirosis contain antigens for *hardjoprajitno* not *hardjo-bovis*, the type most commonly isolated in North America [5]. Challenge studies evaluating the efficacy of pentavalent vaccines for protection against *hardjo-bovis* have yielded conflicting data; however, these studies utilized different products and the time period from initial vaccination until administration of the booster differed greatly [7, 8]. Administration of a commercial monovalent *hardjo-bovis* vaccine has been shown to prevent renal colonization and shedding of *hardjo-bovis* following challenge [9]. The subject is further complicated by the true incidence of infertility that can be attributed to *hardjo-bovis*. A study in California involving 207 first-lactation cows found that cows seropositive for serovar *hardjo* had a mean time from calving to conception 34 days longer than seronegative cattle [10]. However, when beef cattle were vaccinated with a commercially available monovalent *hardjo-bovis* vaccine and administered oxytetracycline to eliminate the carrier state of *hardjo-bovis*, no improvements in pregnancy and calving rates were observed [11]. The decision to vaccinate and the product used should be based on the prevalence of leptospirosis in the area and its potential impact on reproductive performance in the herd. All the commercial vaccines currently available are killed products which should be boostered according to the label and revaccination annually is recommended.

Campylobacteriosis

Bovine venereal campylobacteriosis caused by *Campylobacter fetus* subsp. *venerealis* results in transient infertility in cattle and occasional abortion. The bull serves as an asymptomatic carrier transmitting the organism to cows during coitus. Vaccination of bulls with an oil-adjuvanted killed bacterin can be both protective and curative [12–14]. Exposure of 4- and 5-year-old vaccinated bulls to *C. fetus*-infected heifers resulted in transmission to only 1 of 17 unvaccinated naive heifers and carrier status was not established in any of the five bulls exposed [12]. Therapeutic vaccination of experimentally infected 5-year-old bulls with a two-dose series of an oil adjuvanted killed bacterin was curative in 8 of 10 bulls [13]. While prophylactic vaccination will not clear infection in all bulls, it is an important and inexpensive method of control. The efficacy of vaccines containing an aluminum hydroxide adjuvant is questionable [14, 15]. All bulls should be vaccinated and boostered according to the vaccine label and annual revaccination is recommended.

Bovine Viral Diarrhea

Clinical manifestation of BVDV infection in cattle can vary from subclinical to fatal [16]. BVDV is transmitted primarily by direct contact with bodily fluids of infected cattle. Venereal transmission via semen from acutely and persistently infected bulls is also well documented [17, 18]. Cattle that are born persistently infected with BVDV shed large amounts of the virus and are considered the major reservoir. Control of BVDV centers around elimination of these persistently infected individuals combined with vaccination and biosecurity measures to prevent exposure to BVDV [16].

The bull is also capable of maintaining a prolonged testicular infection that persists beyond the initial viremia [19, 20]. These localized prolonged testicular infections can persist for at least 2.75 years and have been induced by vaccination of seronegative peripubertal bulls with a modified live vaccine containing non-cytopathic BVDV [20, 21]. Semen from one bull with prolonged testicular BVDV infection was capable of infecting heifers through artificial insemination (AI); however, others studies evaluating transmission from bulls with prolonged testicular infection have not resulted in transmission [20, 22]. While the exact risk of transmission from these bulls appears to be low, further studies are needed before the use of vaccines containing non-cytopathic BVDV can be recommended in breeding bulls [14, 20]. Current recommendation are to vaccinate all bulls with a vaccine containing cytopathic modified live BVDV at least 28 days prior to breeding and booster according to the product label recommendations [14].

Infectious Bovine Rhinotracheitis

Bovine herpesvirus (BHV)-1 is a common cause of abortion, conjunctivitis, and respiratory disease of cattle. Genital infections also occur, resulting in balanoposthitis of bulls and pustular vulvovaginitis of cows. Cattle that become infected often develop latent infection that can reactivate later, complicating BHV-1 control in the herd. The virus is shed by the mucous membranes of the respiratory tract, conjunctiva, and genital tract and transmission occurs primarily through direct contact. Venereal transmission occurs during natural mating and through AI with contaminated semen [23].

Vaccine products containing killed or modified live BHV-1 are available for cattle. In general, the protection induced by modified live viral products is more rapid and of longer duration than killed viral products. However, modified live BHV-1 vaccine products are capable of establishing latency that can be reactivated under stress and have been reported to cause abortion if used inappropriately in pregnant cows [24–26]. Most modified live products available for use in pregnant cows carry a label which states that the vaccine may be used in pregnant cows and calves nursing pregnant cows only if those cattle were vaccinated with the same product within the last 12 months. In order to err on the side of caution, it is my opinion that bulls should be vaccinated with the same BHV-1 vaccine product as the mature cow herd. Vaccine products containing modified live BHV-1 should not be administered to bulls unless the cow herd received the same vaccine product in the previous 12 months.

Trichomoniasis

Trichomoniasis caused by the protozoan *Tritrichomonas foetus* subsp. *venerealis* is a venereal disease resulting in endometritis, embryonic death, abortion, and pyometra in infected cattle. The bull serves as an asymptomatic carrier [27]. Prevention of bovine trichomoniasis is centered on biosecurity practices to avoid introduction of infected individuals into the herd. Only one *T. foetus* vaccine is currently available in the USA. Administration of this killed vaccine to bulls aged 55–72 months prevented colonization of the preputial epithelium following intrapreputial challenge [28]. Within affected herds, vaccination of bulls and cows may reduce the economic impact of this pathogen [14].

Breeding Period

The length of the breeding period is generally determined by the producer and can vary from 65 to 365 days. A controlled calving/breeding season has obvious economic advantages and simplifies the overall health management of the herd; however, in many herds especially in the southern USA the bull is housed with the cow herd year round. The following discussion on the stocking rate of bulls will assume that the herd has a controlled breeding season of 65–90 days.

Stocking Rate

The correct stocking rate of bulls, or more specifically the bull to cow ratio that will result in the most efficient and successful servicing of females, is not known. Typical bull to cow ratios in the USA ranges from 1 : 20 to 1 : 30 for mature bulls [29]. Young bulls less than 3 years of age can be placed with one cow per month of age; for example, a 15-month-old bull can be expected to service 15 cows during the breeding season [30]. Assuming that a bull is fertile and has passed a breeding soundness examination, other factors that will influence the number of females he can successfully service during the breeding season include his libido or "sex drive" and his social ranking, both of which are difficult to quantify and predict. The assessment and measurement of bull libido can be attempted by performing a servicing capacity test; however, no standardized testing method exists and results vary depending on age of the bulls tested, breed, and the methodology of the test [31]. An Australian trial of 1100 servicing capacity tests found that measurements of sexual behavior were not consistently correlated to the calf output of the tested bulls and that the main value of a servicing capacity test may be to identify bulls that are unable to mount and/or gain intromission [32].

Social dominance occurs in multisire herds where the presence of a dominant bull suppresses mating activity of other bulls. Dominance is generally expressed by older, more senior bulls and the effects may become more pronounced as the number of females assigned to each bull is reduced [29, 33]. The impact of social dominance on individual bull performance was demonstrated by a series of trials from northern Australia which examined the effect of the bull to cow ratio in multisire breeding groups on reproductive performance. Reducing the percentage of bulls to cows from 6 to 2.5% had no impact on conception patterns between groups. Variation in calf output between bulls was noted in both groups but was significantly reduced when fewer bulls were utilized. The area of movement of individual bulls within the pasture expanded as the percentage of bulls to cows decreased, suggesting that the effects of social dominance were reduced by increasing the breeding pressure on the bull battery. Interestingly, bull attrition due to injury was also reduced [33].

Recently, workers from Auburn University published the following formula for bull to cow ratio:

$$B = 1 + \left[\left(T - N \right) / 1 / 2N \right]$$

where B is number of bulls required to breed the given number (T) of cows in the breeding group and N is the individual bull's fair complement of cows as a single-sire breeding group. N is calculated for bulls less than three years of age by assigning one cow per month of age. Scrotal circumference is used to calculate N for bulls greater than three years of age, where one cow is assigned for each centimeter of scrotal circumference.

This formula assumes that after the first bull each additional bull added to a multisire herd can only be expected to reliably service half the number of females that he would service in a single-sire breeding group [30]. Utilizing this formula for a 200-cow breeding group will demonstrate the inefficiency that many people believe exists in a multisire breeding group. The calculated requirements for this group would be nine adult bulls with 40-cm scrotal circumferences; however, if this same group was divided into single-sire breeding groups of 40 cows each, only five bulls would be needed to service the entire herd. Clearly, single-sire breeding groups would be a more efficient use of bulls in this situation, but the added expense of fencing required for single-sire breeding groups and the risk of bull injury/illness going unnoticed make producers hesitant to adopt this management strategy.

Scientific recommendations on bull stocking rates are difficult because variations in libido exist between bulls but are hard to predict and the effects social dominance on breeding performance are not well understood. While the effects of social dominance and breeding overlap may reduce the efficiency of multisire breeding groups in terms of the number of bulls needed compared with single-sire breeding groups, these effects have been reportedly reduced by increasing the breeding pressure on the bulls [33]. Clearly, more research is needed in this area so that more accurate recommendations can be made to producers, allowing them to avoid purchasing and maintaining excess bulls. Until further research is published, the Auburn formula provides a good guide when making recommendations to producers and is not likely to result in inadequate bull power.

Producer Observation

Libido is not assessed during the routine breeding soundness examinations of bulls, so it is up to the producer to ensure that the bull is actively servicing cows. Producers should take care to observe that intromission is achieved when a female is mounted and make note of the date when the individual was serviced. Breeding injuries and lameness are best identified early so that treatment can be initiated and adjustments made before the herd's reproductive performance is reduced. Routine observation of the herd and documentation of breeding activity during the breeding season is especially important in the single-sire breeding group to prevent loss or reduction in the calf crop.

Non-Breeding Period

The non-breeding period provides an opportunity for the bulls to rest and replace any weight that was lost during the breeding season. Body condition scoring or weighing bulls at this time will allow producers to make the necessary dietary changes to ensure that the bull battery enters the next breeding season at an appropriate body condition. The effect of body condition on breeding performance of mature bulls is not well documented; however, logic would suggest that avoiding overconditioning will reduce the effort to mount and the force on the hindlimbs during mating. Commonly, breeding bulls are fed to reach a body condition score of 6–7 prior to entering the breeding season [34]. Dietary energy excess has been shown to have detrimental effects on semen quality in yearling bulls. These effects are thought to be related to impaired thermoregulation of the testes due to increased scrotal fat deposition [35].

Housing during the Non-Breeding Period

Group housing is generally recommended for bulls used in multisire breeding groups to reduce bull attrition due to injury during the breeding season by allowing the social hierarchy to be established and maintained in the absence

of females. Some separation of bulls may be necessary depending on the uniformity of the bull battery. Young bulls less than two years of age may need to be grouped separately to avoid competition for feedstuffs and allow for continued growth and development. Bulls finishing the breeding season in poor body condition may likewise be grouped together or fed separately until their body condition improves. Housing recommendations in cold climates with significant snowfall may also include the addition of a shelter with bedding to avoid scrotal frostbite.

Parasite Control

Specific recommendations on parasite control will vary depending on the geographical region. Most bulls are dewormed once yearly prior to the breeding season. In warmer more humid climates, twice-yearly deworming may be necessary, especially in younger bulls. Yearling bulls are at the highest risk for internal parasitism and may need to be dewormed immediately following the breeding season to remove the parasite burden accumulated while in the cow herd. Fly control is also important in bulls to eliminate fly strike in the scrotal region, which can lead to inflammation and impaired thermoregulation of the testes [34].

Biosecurity Concerns for Replacement Bulls

The introduction of replacement bulls into the cow herd represents a significant risk for disease introduction. The primary pathogens of interest in the USA include *Brucella abortus*, *Campylobacter fetus* subsp. *venerealis*, *Tritrichomonas foetus* subsp. *venerealis*, *Leptospira* species, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium bovis*, BVDV, and BHV-1.

In addition to diagnostic screening for potential pathogens, all replacement bulls should be quarantined for a minimum of 30 days before exposure to the cow herd. This period will allow viral shedding to cease in bulls with acute BVDV and BHV-1. Producers should also be aware that no diagnostic test is 100% sensitive and therefore bulls should be purchased from herds with good health programs. Purchasing bulls from herds that are certified free for a specific pathogen provides additional security. Likewise, purchasing virgin bulls eliminates the risk for *C. fetus* and *T. foetus*.

Recommendations for Dairies Utilizing Natural Service

The genetic improvements associated with AI and their impact on production and profitability in the dairy industry are well established. However, utilization of natural service (NS) is still widespread for a variety of reasons [36]. Bulls may be utilized to improve estrus detection, resulting in increased conception rates, as a management tool to prevent dystocia in heifers, for the production of dairy beef, and to decrease the cost and labor associated with AI [36, 37]. The use of NS to improve reproductive efficiency in dairy herds with poor reproductive performance can improve profitability by decreasing the calving to conception interval, resulting in increased milk production per cow and reducing culling. These improvements may not be economically significant in herds with acceptable reproductive performance [37]. The most obvious disadvantages of NS are reduced genetic improvement, cost associated with bull purchase and replacement of bulls, disease introduction and transmission, and safety. The extent of the reduction in genetic improvement by utilizing NS depends on the quality of bulls utilized. Some dairies offset this loss by utilizing a combination of one or multiple attempts of AI followed by NS. Only heifers produced from AI are then retained for replacements.

The typical stocking rate of bulls on dairies ranges from 1 : 20 to 1 : 30 bulls to cows [38, 39]. This stocking rate can vary significantly between dairies, especially those that utilize AI followed by NS. In herds that utilize this combination, the bulls needed should be based on the actual number of cows that are eligible to become pregnant at the time of bull turnout. The length of the bull breeding season is also quite variable between dairies and is commonly impacted by seasonal breeding. Dairy bull attrition due to lameness is a common problem resulting from a combination of diet, housing and flooring, and injury from fighting. Lactating cow rations are higher in energy, protein, and calcium, which exceed the needs for bull maintenance. The excess energy leads to laminitis, while excessive calcium has been shown to induce skeletal lesions [38]. Rest and rotation of bulls during the breeding period is recommended to reduce the impact of diet and hard-surface flooring on lameness. Whether bulls are rotated or not, the length of rest periods varies greatly among dairies. One report from California dairies utilizing NS found that bulls are never removed from the breeding group after initial introduction in 47% of the dairies surveyed [40]. Reports from Australia found that bulls are left in the breeding group from 1 to 14 days and then are rested for an average of 7 days before returning to the breeding group. The Australian dairies surveyed used bulls on average for 56 days following multiple attempts at AI [41]. Using young bulls, less than 2.5 years of age, is recommended to reduce the impact of social dominance on breeding and fighting and to improve human safety. Social dominance is thought to be less dramatic if bulls of similar age and size are utilized [36].

Other management considerations of paramount importance include the fertility of the bulls used and their potential to introduce disease into the herd. Lameness is common in dairy bulls, as discussed above, and it can impact fertility by reducing bull libido and physical ability to seek out and service cows. Chronic lameness will also reduce semen quality. Dairy bulls should be assessed frequently for lameness and should have breeding soundness examinations performed before introduction and, at least, annually thereafter [36]. The biosecurity risk that the dairy bull represents is similar to the concerns discussed above regarding beef bulls. Special considerations should be given for sexually transmitted diseases like trichomoniasis, campylobacteriosis, and host-adapted leptospirosis when NS is utilized. The diagnosis, treatment, and prevention of these conditions is covered in their respective chapters. Likewise, a complete overview of biosecurity measures for the beef and dairy herds is covered in Chapter 28.

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12

Management of Bulls at Custom Collection Studs

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Veterinary Services for the Commercial Bull Stud

The semen collection – or "custom bull stud" – industry has markedly evolved during the time it has been in existence. Initially, a large majority of semen product was provided by large cooperatives or studs that maintained a large resident bull herd, catering primarily to the dairy industry and its needs. Most bulls in these studs were either purchased outright by stud management or secured by a long-term lease, and thus breeders/owners very rarely realized the full rewards of their production successes. Beef cattle were processed as an aside, as the beef industry's use of frozen semen products was not widespread. As technology and breeding synchronization systems have evolved, the demand for semen in the beef industry has skyrocketed and custom bull studs have developed throughout the USA. These custom studs allow bull owners the opportunity to present herd sires with proven genetic merit, process and store these quality genetics, and retain ownership and possession of the bull. Also it gives bull owners the option to access the international marketplace by allowing them to transport the frozen semen product anywhere in the world.

To assure health quality of the frozen semen products, any semen produced for export must meet standards set by Certified Semen Services, Inc. (CSS). Recommendations for housing, collection, handling, and health testing are made and supervised by administrators within this organization. Recommendations for health standards for residents at the bull studs are taken from guidelines published by the World Organization for Animal Health (OIE). This group concerns itself with advising importing and exporting countries on protecting themselves from introducing disease into their countries from foreign sources. Many countries will take the guidelines of the OIE and formulate entry requirements for cattle as well as bovine germplasm, both semen and embryos. Guidelines are specific and must be followed, as entry is forbidden unless standards are met.

The Veterinarian's Role in the Bull Stud

The primary role of a veterinarian in an artificial insemination center (AIC) is to ensure biosecurity and animal welfare for the bovine population housed there. Standards for testing protocols are established and readily available on the website of the National Association of Animal Breeders (NAAB) (www.naab-css.org). These are standard recommendations for the industry that are accepted by the United States Department of Agriculture (USDA)/Animal and Plant Health Inspection Service (APHIS) but certainly may be augmented by any further testing considered appropriate by the center's veterinarian.

The ultimate responsibility of the veterinarian is to assure the welfare of the animals under his or her care. Since the stud is usually compensated by the units of semen provided, it is in the stud's best interest to ensure that the cattle in its charge are adequately cared for. This means that feed, water, forage, and facilities meet the best of standards for quality. Although some facilities may retain the services of a nutritionist, others may rely on their veterinarian for suggestions on ration formulation or forage sampling and testing. Supplementation of minerals should not be forgotten for long-term resident herd members. Quality control of commercial feed rations should be observed and the resident veterinarian should periodically check feed and storage facilities to ensure proper care and quality.

Since the focus of this chapter is the private veterinarian consulting for the bull stud, we should concentrate on the start of the process: entry into the bull stud. There are three periods at which health testing requirements must

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be performed: pre-isolation, isolation, and routine testing that will take place at regular intervals after entry into the resident herd.

The first round of testing, pre-isolation testing, may be done at the farm of origin by the resident veterinarian. Pre-entry requirements should be met within 30 days prior to entering the isolation facility. If bull owners do not wish to trouble themselves with testing, many custom studs offer pre-isolation facilities to house the bull while testing is conducted. These pre-entry requirements also apply to any mount or jump animals to be used in isolation or the resident herd. The isolation and resident testing requirements will be conducted upon arrival at the collection facility and at prescribed intervals thereafter. All testing phases require a complete physical examination, a negative intradermal test for tuberculosis, a negative test for bovine brucellosis, and a negative test for bovine leptospirosis. Those with titers of 1:100 or greater for leptospirosis may be reevaluated in two to four weeks. Those with titer no greater than 1:400 will be considered stabilized and allowed entry into isolation. Testing for bovine viral diarrhea virus (BVDV) should be performed before entry and a negative result obtained; the testing must only be conducted by virus isolation from whole blood or serum performed in cell culture, followed by evaluation of cell cultures with immunoperoxidase (IP), fluorescent antibody (FA), enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction (PCR). Lastly, testing for campylobacteriosis and trichomoniasis is required during the isolation period and at six-month intervals thereafter. For both diseases, bulls are required to undergo a series of weekly tests, with the required number varying depending on the age of the bull. Table 12.1 outlines a basic AIC testing protocol. Please refer to http://www.naab-css.org

Table 12.1 Basic AIC testing protocol per CSS testing requirements.

	Testing environment		
	Pre-entry to isolation	**Isolation**	**Resident herd**
Physical examination	Conducted by accredited veterinarian	Conducted by accredited veterinarian	Conducted by accredited veterinarian
Tuberculosis	Negative intradermal tuberculin test (within 60 days prior to entry)	Negative intradermal tuberculin test at least 60 days after pre-entry test	Negative intradermal tuberculin test at 6-month intervals
Brucellosis	Official test of state where bull is located. Blood serum test (CF, BAPA, or Card)	CF and one BAPA or Card test at least 30 days after pre-entry testing	CF and one BAPA or Card test at 6-month intervals
BVDV	One negative virus isolation test performed on either whole blood (animals less than 6 months of age) or serum	Virus isolation and serologic testing at least 10 days after entry into the isolation facility. If seropositive, virus isolation of semen required	Not required
Leptospirosis	Blood test for five serotypes important in the USA[a]	Blood test for five serotypes important in the USA[a] at least 30 days after pre-entry test	Blood test for five serotypes important in the USA[a] at 6-month intervals
Campylobacteriosis	Not required	Series of negative culture tests of preputial material or screening by FA, with any positive FA tested by culture for final determination; 1, 3, or 6 consecutive weekly tests; number of necessary tests dependent on age	Negative single culture test of preputial material or FA for screening test at 6-month intervals
Trichomoniasis	Not required	Series of negative microscopic examinations of cultured preputial material; 1, 3, or 6 consecutive weekly tests; number of necessary tests dependent on age	Negative single microscopic test of cultured preputial material at 6-month intervals

Source: From CSS Minimum Requirements for Disease Control of Semens Produced for AI, January, © 2014, National Association of Animal Breeders.

BAPA, Buffered acidified plate antigen; BVDV, bovine viral diarrhea virus; CF, complement fixation; FA, fluorescent antibody.

[a] *Leptospira pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, *L. grippotyphosa*.

for complete details and the most recent updates regarding testing requirements.

Examination of entry-level animals should be conducted as soon as possible after admission to pre-isolation. A good physical should include observation of ambulation on a surface that provides good footing, preferably loose soil or grass. At this time all physical features should be assessed from the muzzle to the tail, paying particular attention to the feet (Figure 12.1). After this initial examination, the bull should be properly restrained in a squeeze chute, head gate, or lock stanchion. For beef bulls, a squeeze chute would be most desirable for restraint. Starting at the head, the oral cavity should be examined, making sure that all teeth are present and showing even wear (particularly important in more aged herd sires). The eyes should be evaluated for clarity, any presence of neoplasia, or previous injury. Make sure both eyes respond to movement and function normally. The ears are checked for any unusual discharge; in some areas of the country they should also be checked for ear ticks. The bull's chest should be auscultated and any abnormalities noted, as should the entire abdominal cavity. Particular attention should be paid to rumenal contractions, both in strength and number. Several peripheral regional lymph nodes are palpated for any evidence of lymphadenitis. Next, the underline of the bull should be evaluated from the bottom of the chute. The preputial orifice is palpated and examined as well as the scrotum, with palpation of the testes and all epididymal structures. Record the scrotal circumference with a scrotal measuring tape. Evaluation of the lower limb joints and hooves is performed at this time as well. Lastly, rectal palpation should be performed to evaluate abdominal structure, with a rectal temperature being taken and recorded. It is vital to pay attention to accessory reproductive organs, with specific interest in the health of the seminal vesicles. During the rectal examination, it is a good idea to massage the crus of the penis to allow an assistant the opportunity to fully extend the penis. Both vaginal rings are palpated and any abnormalities noted. After examination and pre-entry testing, follow the recommendation of the NAAB/CSS for entry into isolation after presentation at the stud. Some producers may not be interested in submitting a bull for further testing as they have no desire to export semen. It is always recommended to follow CSS guidelines on any mature bull that has been breeding cows before presentation for collection.

At the time of entry into isolation at the bull stud, or AIC as some like to be called, the bull is assigned a unique sire code number per the NAAB uniform coding system, if the stud is an NAAB participant. This code is separate and apart from any tattoo or brand used in a breed registry. The bull may display this code in a number of ways but most AIC's use ear tags with the code plainly marked. This code can be used by the center to identify product produced by the bull, although other permanent identification is used for health testing purposes (i.e. hide brands or tattoos).

After admission the bull entering the isolation facility must go into a pen that has been properly cleaned of any residual organic material deposited by the former occupant and disinfected (Figure 12.2). For stalls with dirt floors, a disinfectant applied as a mist over the area should be sufficient to meet NAAB standards. The veterinarian should be aware of the facilities and any damaged portions of housing that could injure an animal. The isolation facility must be separate and apart from the resident housing. All equipment used for cleaning, feeding, watering, and semen collection of the isolation bulls must remain separate from the resident herd. This includes feed storage and any mechanical means of transport. Workers must change



Figure 12.1 Bulls going into a chute.



Figure 12.2 Ideal conditions for housing bulls in central Texas, Elgin Breeding Service.

boots or protective cover before entering or leaving an isolation area and going to other quarantine areas. Testing on the isolation bulls starts with an intradermal test for tuberculosis performed at least 60 days after the pre-entry test; a test for bovine brucellosis not sooner than 30 days after the pre-entry test, with negative result; and a test for bovine leptospirosis not sooner than 30 days after the pre-entry test, with negative result or subject to stabilized titer of less than 1 : 400. Bovine campylobacteriosis requires up to six cultures and examination of preputial wash material collected at weekly intervals. Bovine trichomoniasis has the same weekly culture requirement during the isolation period. Testing for BVDV is performed no longer than 10 days after entry via whole blood or serum, with evaluation by FA, IP, or PCR on cell culture. All bulls having semen collected will have future testing for BVDV before semen is released. Semen testing by PCR is preferred, with at least one passage through cell line culture.

Veterinarians should also familiarize themselves with the diagnostic laboratory they will be working with, particularly with regard to sample submission requirements, the intricacies of testing procedures, and especially testing protocols and the reporting of test results, as understanding results is very important. Any positive test results, particularly on pre-entry tests, should be evaluated in conjunction with previous vaccination history. Frequently, certain vaccines produce a prolonged immune response that may interfere later on with attempts to approve semen for export. Leptospirosis vaccination produces a persistent titer for prolonged periods in certain cases.

Once the bull meets all the requirements of the isolation protocol, he is allowed to enter the resident herd, where testing is continued to assure compliance with international protocols. The required testing is preformed semi-annually on each bull occupying the resident herd. Previous testing is continued at a less frequent pace (every six months), with tuberculosis, brucellosis, leptospirosis, trichomoniasis, and campylobacteriosis all evaluated using the same testing protocols as discussed earlier. Bulls must be restricted from any contact with any other cloven hoofed animal; if such contact occurs, the bull must be returned to isolation and the testing protocol for isolated bulls must be followed once again.

Facility overview is another responsibility of the veterinarian. Supervision of the artificial vagina (AV) preparation room, collection area, bull housing, and semen lab and processing area should all be evaluated for cleanliness, absence of any cross-contamination, and ability to disinfect contact surface areas. Semen processing equipment should be clean and serviceable and packing and shipping areas clean and organized (Figure 12.3). The veterinarian is responsible for all these areas, as noted when signing off on any federal export certificate. Accuracy is the goal of the veterinarian to assure that standards set by the OIE and NAAB/CSS are met to ensure continued access to all markets.



Figure 12.3 Semen canisters for storage of frozen semen and embryos. Note the cleanliness and organization of the facility.

The Collection Process

The collection of semen for cryopreservation is a fairly simple and straightforward process, except for those with no experience. Therefore the procedures should only be administered by someone who is competent and experienced at handling bulls and very familiar with the collection process. Good facilities are paramount to a successful collection attempt, particularly when dealing with beef bulls that have primarily had pasture breeding experience. Collection of semen from bulls with an AV is not for the faint of heart. The procedure basically involves allowing the donor bull to mount or "jump" another steer, bull, or cow and diverting the penis laterally in order to introduce the extended penis into the AV. Usually, once bulls are familiar with the technique, they will readily mount and serve the AV. Training them to do this is an art, particularly when dealing with less docile mature beef bulls and bucking bulls.

CSS mandates guidelines to be followed for bull health and cleanliness. Steers are the most common mount animal unless the facility prefers the use of a phantom (dummy structure designed for supporting a mounting bull). Using cows as mount animals is discouraged because of the potential for penetration and contamination by the bull. Mount animals should have their hair closely clipped from the hindquarters, allowing for ease of cleaning and disinfection, which should be accomplished after every use. The process usually requires at least two people and

preferably three. One person is assigned the mount animal, which is usually haltered or handled by nose ring. This person's responsibility is to position the mount animal for mounting and to assist in keeping it in position during collection. In large facilities there is also a bull handler who may have the bull haltered or tethered to control him during the mounting process. Most beef bulls in custom studs are free to mount the jump animal. Lastly, the semen collector manages the AV and is in charge of the entire collection area (Figure 12.4). It is this person who ensures the sample is properly identified and collected hygienically and is responsible for the safety and welfare of the team as well as the animals.

The procedure of semen collection is rather straightforward. Several bulls are usually penned separately along a "bull alley" facing the collection area. Mount animals are allowed to move along in front of the bulls in order to initiate some arousal. The bulls are allowed to observe other bulls mounting and being collected from this vantage point. It can be very evident that bulls in the bull gallery are aroused by the amount of vocalization that occurs as a bull is being collected.

Once it is time for a bull to be collected, he is allowed to leave his pen and enter the mount area. At this time, the mount handler will move the mount animal to prevent the bull from mounting. This may continue for several minutes until such a time as the semen collector is satisfied the bull is properly aroused. The semen collector may further determine that the bull needs to "false mount" to further his arousal and thus increase the quality and quantity of semen he produces. This is accomplished by the bull handler allowing the bull to mount but the semen handler diverting the penis away from the mount animal as the mount is withdrawn from under the bull. As discussed previously, when the semen collector believes the bull is properly stimulated, the bull is allowed to mount for collection (Figure 12.5). After collection the bull is either returned to housing or is placed back in the bull alley for another collection attempt.

The preparation of the AV is important as the tactile sensation received by the bull is paramount to his semen production. It should simulate the natural breeding act as closely as possible. The AV is constricted, with a ridged tube that has a port allowing water to be added between the rigid structure and a latex liner. Within this liner another is placed to facilitate collection of the semen from the bull. This liner has a tapered end that allows the inclusion of a glass or plastic receptacle that will serve to hold the collected semen sample. Water added to the water jacket should be in the temperature range 40–60 °C and varies according to the preference of the bull; this has to be determined by the semen collector. Also, the amount of water within the jacket determines the rigidity of the liner, which can impact the bull's reaction to the AV. It is very important that a sterile non-spermicidal lubricant be applied to the upper one-third of the AV. This improves sensation and reduces the possibility of abrasive injury to the bull's penis.

For those bulls that are too fractious or are incapacitated and unable to jump a mount animal, the collector has the option to use an electroejaculator. Although it is discouraged in the semen production industry, it may be used when all else fails. Usually the bull will produce a slightly less concentrated ejaculate and with the potential for a somewhat higher abnormal sperm count. However, with proper stimulus (rectal massage of the ampulla and seminal vesicles) adequate semen may be collected for processing. Massage of the pelvic genitalia alone usually does not produce adequate semen product for processing and is discouraged in the industry.



Figure 12.4 Collection area.



Figure 12.5 Proper technique for collection of bull semen.

Common Problems of Bulls at Stud

Lameness

Lameness is the most common problem in bulls at stud. The primary complaint from the stud manager will be lameness of acute onset. Some of the more common insults are hoof or foot rot, hairy heel wart (bovine dermatitis), hoof abscess, hoof lacerations, broken toes, and overgrown toes. Hoof abscesses due to wall separations ("white line disease") are most often identified as the problem in these lame bulls. Most white line disease can be traced back to subclinical laminitis occurring some time in the bull's yearling development. The best curative approach is to access a hydraulic tilt table and to place the bull in lateral recumbency in order to remove the damaged sole and/or wall (Figures 12.6 and 12.7). Feed and water are withheld for 24 hours before table placement to reduce the chance of rumenal tympany while in lateral recumbency. If over 1 cm of sole or hoof wall is removed, it is recommended that an elevated block be placed on the opposing toe in order to reduce contact with the ground by the injured toe. Postprocedure antibiotics and analgesics are necessary to prevent infection and pain due to the lesion. Other injuries causing lameness are usually accidents that can occur in the collection areas, for example shoulder "sprains" when bulls get their forelimb hung up on the back of the mount animal and stifle injuries when mounting or dismounting the mount animal. Sometimes the only method available to properly diagnose the problem is diagnostic anesthesia, which often requires the use of a tilt table. Superficial injuries may be treated with a few days of non-steroidal anti-inflammatory drugs. The more serious may require more aggressive measures.



Figure 12.6 A well-designed work area for performing lameness examinations at a custom stud. Note the hydraulic tilt table and footbath in foreground.

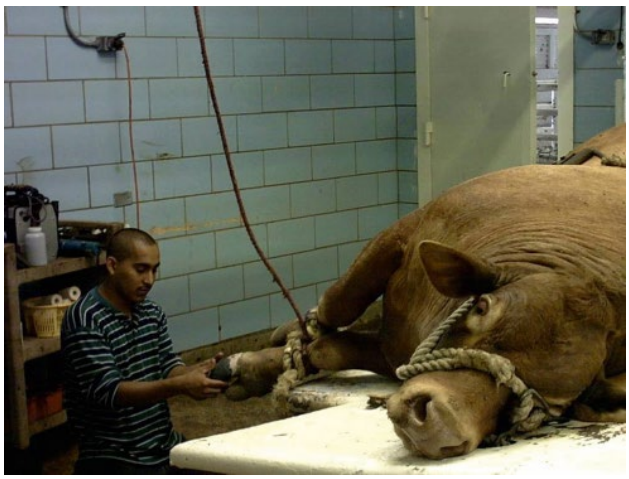


Figure 12.7 Assisting in preparation of hoof block application.

Treatment of issues such as foot rot or hairy heel wart may best be treated via footbath or topical therapy. The more stubborn infections may require antibiotics and periodic topical antibiotic bandages. If lameness persists after a few days of therapy, it is always recommended to question the original diagnosis and reinvestigate the problem.

Occasionally, a diffusely swollen limb is observed associated with a joint (most often a tarsus) with accompanying pyrexia, which can signal initiation of septic arthritis secondary to a generalized bacteremia. These are usually consequences of some kind of stress, most often change of feed or feeding methods. Any bull presenting with generalized cellulitis in a limb should be considered a medical emergency. Most bulls are not weight-bearing and can be difficult to deal with in local settings, so it is often better to refer these cases to a facility capable of handling this kind of care.

Penile Injuries

One of the more common penile injuries observed in the bull stud is preputial laceration/avulsion at the site of the preputial reflection. This injury occurs as a friction "tear" due to misapplication of the AV as it is placed on the bull to collect the semen sample. Most often, the only evidence of injury initially will be drops of blood in the AV or in the ejaculate, or by blood dripping from the preputial orifice. This is to be considered a medical emergency and should be addressed as soon as possible after injury. After a pudendal nerve block and/or local anesthesia, the wound should be debrided with copious lavage of the site. Debridement should be followed by suturing with simple interrupted sutures or by surgical stapling. Bandaging of the orifice to maintain the penis in the sheath, with antibiotics and anti-inflammatory drugs, will be required for several days. After recovery, the bull

should only be collected with an electroejaculator or rested from sexual activity for about four weeks.

Occasional preputial lacerations from self-trauma may occur. It is not uncommon to see bulls "catch" the prepuce with a dew claw as they start to rise. These may only require antibiotics and topical medication instead of debridement and surgery. Each case should be independently assessed. Occasionally, the injury may scar sufficiently to reduce the diameter of the prepuce and impede full extension of the penis. If this occurs a penile reefing or circumcision may be required in the future.

Gastrointestinal Tract Problems

AICs will need veterinary services to treat a wide variety of gastrointestinal problems. Most are simple ruminal tympanies or bloat, followed by non-specific and transient diarrheas. Most are thought to be due to mistakes in feeding protocols but on occasion may be caused by changes in gastrointestinal motility brought on by a host of other primary problems. These incidents will respond to systemic treatment of laxatives, oral fluids, and analgesics if warranted. More serious gastrointestinal problems encountered include hemorrhagic bowel disease, perforating abomasal ulcers, intestinal intussusception, and intestinal volvulus. Once diagnosed, these are best referred to those most experienced in dealing with such problems as these bulls will need surgery and intensive aftercare.

Occasionally, an acute disease due to a foreign body may occur in a bull at an AIC. Of course, the penetrating foreign body could have been present for years; such subtle problems should not be overlooked when making a differential list. Advising a custom stud owner on per-os administration of rumen magnets should be considered when creating entry requirements.

Urinary Tract Problems

Urinary tract problems are rare at stud but do occasionally occur. Initial presentation of a bull with urinary obstruction is much like any other abdominal problem: colic-like symptoms, kicking at abdomen, elevated heart rate, increased salivation, straining attempts to defecate and urinate, etc. Most often bulls may respond to analgesic therapy along with smooth muscle relaxants and urinary acidification. It is to be remembered that if the urinary obstruction is relieved, there are probably still many calculi left in the bladder. When urinary calculi are involved it is wise to diagnose the type of calculi present and adjust therapy accordingly, maintaining the bull on acidification until the calculi significantly decrease in a urine sample. In some areas, calculi do not respond to urinary acidification, presenting the clinician with a whole new set of problems. Most bulls that have calculi problems also have an ongoing cystitis requiring bacterial culture and sensitivity testing and prolonged antibiotic therapy.

Summary

Consulting for an AIC can be rewarding both financially and professionally. Many breeders working with an AIC are the elite within their breed and by exposure to the veterinarian through association with the bull stud new client relationships can develop. Consultation services, from disease prevention to improving reproductive efficiency and development, can occur through contacts made with the AIC.

Acknowledgment

The author wishes to acknowledge his mentor Dr. Wallace Cardwell for his countless contributions to the information found in this chapter.

13

Testicular Degeneration

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Introduction

Testicular degeneration may be temporary or permanent; temporary degeneration is much more common and, with removal of underlying cause, testis structure and function often recover. It may be difficult to distinguish between degeneration and a severe disturbance of spermatogenesis, with a marked decline in semen quality. The term "degeneration" is somewhat arbitrary, but usually implies reduction in scrotal circumference, testes may be soft, and sperm quality is poor (usually <20% morphologically normal). With temporary degeneration, there is loss of germinal cell layers near the lumen in many or all seminiferous tubules. Sertoli cells and spermatogonia, which are more resistant to damage, are likely to be retained and if the cause of degeneration is removed, tubules are repopulated with spermatocytes and spermatids, restoring testis size and semen quality. Advanced degenerative changes causing permanent damage include spermiostasis, tubular mineralization, granuloma formation, thickened basement membranes, and fibrosis in focal or diffuse areas of testicular parenchyma.

Pathogenesis

Factors involved in pathogenesis of testicular degeneration include abnormal testicular thermoregulation, nutritional excess or deficiency, toxicity, inheritance, congenital blockage of sperm outflow from testes, infectious disease, severe trauma, and senile atrophy.

Testes of scrotal mammals function normally when testis temperature is 2–6 °C below body temperature [1]. Scrotal insulation is a classic model to induce temporary testicular degeneration [2, 3]. In one study, the scrotum was insulated for 10 days; there were reductions in semen quality, but no fibrotic lesions within 6 months after insulation [4]. Therefore abnormal testicular thermoregulation that is reversible (e.g. obesity) may not be a common cause of permanent damage. Other less common causes of chronic abnormal thermoregulation that may lead to temporary or permanent testicular degeneration include severe chronic scrotal dermatitis, heat and swelling due to orchitis, trauma, chronic illness with prolonged fever, endotoxins (potent gonadotropin inhibitors), a congenitally short scrotum, severe scrotal frostbite leading to adhesions, and incomplete descent of a testis into the scrotum.

Obesity is a leading cause of testicular degeneration in bulls. It is common to fatten bulls for livestock shows and sales. Many bulls become obese and fat accumulates in the scrotum, chronically increasing testicular temperatures [2]. However, after a reduction in body condition, including loss of fat from the scrotum, regeneration of seminiferous tubules may occur with return to normal sperm production [5].

Nutritional deficiencies seldom cause testicular degeneration in bulls. Naturally occurring deficiencies are usually multiple and deficiency of any single nutrient as a cause of bull infertility is rare. Similarly, vitamin deficiencies are also rare as a cause of infertility in larger male domestic animals if forages are normal in quality and quantity. However, reductions in feed quality and/or quantity causing weight loss may depress spermatogenesis via an endocrine effect on developing germ cells rather than by vitamin or mineral deficiencies. In one study, protein supplementation of bulls on poor-quality forage significantly increased dry matter intake, enabling maintenance of live weight, whereas in control (unsupplemented) bulls, weight loss was accompanied by a significant decrease in scrotal circumference and daily sperm production per gram of testis. However, histologically, seminiferous tubule activity was apparently not different between bulls

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with or without protein supplementation [6]. Vitamin A deficiency can cause testicular degeneration in laboratory rats [7], but in bulls under practical feeding conditions, diets that result in long-term marginal vitamin A deficiency or a relatively short-term absence of vitamin A intake probably have minimal effects on spermatogenesis [8]. There is little evidence that deficiencies of vitamins B, C, D, or E cause infertility in domestic animals [9]. Although vitamin E is essential for reproduction in rats, naturally occurring deficiencies resulting in testicular degeneration in livestock have apparently not been reported. Mineral deficiencies may affect male reproduction, but reports are rare. Deficiencies of calcium, manganese, zinc, iodine, potassium, and selenium have not been proven to cause testicular degeneration. Deficiencies of phosphorus, cobalt, iron, zinc, and copper may cause anemia, lack of appetite, and weight loss, with adverse effects on reproduction. Deficiencies of these minerals are often associated with inadequate protein and vitamin A.

Although experimental exposure to various drugs, chemicals, and heavy metals causes testicular degeneration (mainly in rats and mice), there are very few reports of naturally occurring cases in bulls [9, 10]. Ingestion of plant toxins may cause testicular degeneration. *Fusarium* mold in grain crops is common; one of the toxins produced is zearalenone. It has an estrogenic effect, with potential to reduce semen quality and cause testicular degeneration. In one report [11], 23 young rams became infertile after eating grain containing zearalenone (5–20 mg/kg) for several months. Furthermore, two bulls fed maize containing zearalenone (20 mg/kg) for 72 days had poor semen quality after 21 days [11]. Histologically, rams' testes had complete destruction of germinal epithelium and aspermia, whereas in bulls, degeneration of the germinal epithelium was marked only in certain areas of the testes, although >75% of sperm were degenerate [11]. Cottonseed meal may cause gossypol toxicity; it alters mitochondrial structure and function and causes abnormalities of spermatocytes, spermatids, and mature sperm [12], although it did not appear to cause testicular degeneration [13, 14]. Locoweed (*Astragalus lentiginosus*) may cause temporary testicular degeneration, as reported in mature rams [15]. Growth-promoting implants have potential to impair testicular development [16, 17]. When bulls were implanted with zeranol (Ralgro^{TM}) at birth and at 3 and 6 months of age, or every 3 months from birth to 18 months of age, scrotal circumference was reduced but tended to recover with increasing age. However, implanting bulls with zeranol after 7 months of age had limited effects on reproductive organs.

Congenital and inherited disorders of testes and epididymis may be involved in testicular degeneration. Young bulls with testicular hypoplasia may produce semen with satisfactory quality, but are more prone to develop testicular degeneration at two to three years of age [10]. In young bulls, small scrotal circumference has been associated with a lack of germinal epithelium within seminiferous tubules [18, 19]. Hypoplasia may be unilateral or bilateral, although most cases are unilateral and the left side is more frequently affected [10]. There appears to be no clear definition for testicular hypoplasia based on physical measurement. Histologically, testicular hypoplasia is defined based on cell populations in seminiferous tubules. Testicular hypoplasia is congenital and possibly hereditary. For example, in Swedish Highland cattle, it appears to be caused by an autosomal recessive gene with incomplete penetrance [20]. This condition occurs in many breeds, albeit at very low frequency [21]. Double muscling (myofiber hyperplasia), inherited as an autosomal recessive, is associated with a high incidence of bilateral testicular hypoplasia [22].

The embryo needs to connect its gonads with the mesonephric urinary system to develop its excurrent duct system. The connection comes via the rete tubules that develop between the fetal gonad and mesonephros. The ureter for the mesonephros, the mesonephric duct, will become the epididymis. Efferent ductules develop from the mesonephric tubules and join to the mesonephric duct via rete tubules during embryonic development. Failure of some efferent ductules to join with the rete may later (at puberty) result in sperm impaction of blind-ending ductules; their subsequent rupture causes sperm granulomas. If all efferent ductules are obstructed, seminiferous tubule fluid and sperm cannot leave the testes, resulting in testicular enlargement, edema, and degeneration.

Infectious organisms may be involved in testicular degeneration. *Eperythrozoon* infection caused anemia, scrotal and hindlimb edema, and soft testes in young bulls. Loss of scrotal thermoregulation was likely the main cause of testicular degeneration and poor semen quality [23]. In another report, testicular degeneration and loss of libido occurred in beef bulls experimentally inoculated with *Anaplasma marginale* [24]. Testicular degeneration was confirmed by histopathology and semen evaluation. Picornavirus and bovine enterovirus isolated from semen and feces of a bull were implicated as a cause of orchitis, testicular degeneration, aspermatogenesis, and loss of libido [25].

Diagnosis

During clinical evaluations, it may be difficult to distinguish between disturbances of testis function that reduce sperm output and increase sperm abnormalities compared to more severe disturbances of testis function that cause degeneration. Therefore a clinical diagnosis of degeneration is arbitrary. Degeneration does not necessarily reduce sperm concentration in the ejaculate, due to sperm accumulation in ampullae during sexual rest.

Ultrasonography is not necessarily clinically useful for distinguishing between normal testis tissue and tissue that has lost germinal epithelium [26]. When considerable fibrous tissue is present, testes may become more firm; however, a diagnosis of fibrosis is best accomplished with ultrasonography. Echographic anatomy of abattoir-derived bull testes has been reported [27] and ultrasonography of testes does not affect semen quality [28]. Ultrasonography has been used to examine effects of testicular degeneration induced by scrotal insulation in bulls [4, 29, 30]; however, there were no evident visible changes in ultrasonograms after induced testicular degeneration. Furthermore, some studies failed to demonstrate significant correlations between computer analysis of ultrasonogram pixel intensity and semen quality, either during breeding soundness evaluations or after scrotal insulation [29, 31]. In other studies, echogenic changes induced by scrotal insulation preceded increased sperm abnormalities [4]; changes in pixel intensity accounted for 13–25% of variation in semen quality of ejaculates collected two to four weeks after ultrasonographic examination.

Prognosis

Recovery of normal testis tone and spermatogenesis is possible in most cases of testicular degeneration if the cause can be removed. Obese bulls may be fed restricted diets or put on pasture for weight loss, although it often takes three to four months for sufficient weight loss and seminiferous epithelium regeneration to restore semen production. When breeding seasons are short and bull to female ratio is ~1 : 35, there is sufficient breeding overlap to prevent an infertile dominant bull from making a significant impact on herd fertility [32, 33].

Testicular Fibrosis

Focal testicular fibrosis is common in some groups of bulls after weaning. Diffuse fibrosis is much less common and in aged bulls it occurs more commonly in ventral parts of testes [10]. Routine ultrasonography of the testes of bulls may reveal presence of a few to many foci of fibrosis, with or without production of elevated percentages of abnormal sperm. In one study [34], ultrasonography of the testes was done in bulls at three locations in western Canada ($n = 325$) and one in Argentina ($n = 387$) to determine prevalence of fibrotic lesions and relationships between fibrotic lesions and location, age, breed, testis size, and semen quality. Bulls in western Canada are typically fed forage and grain-based rations after weaning, whereas in Argentina, weaned calves did not receive grain supplementation until 18 months of age. Bulls used in the study ranged in age from 3 to 20 months and ultrasonography was done at varying ages.

Bulls in all groups in Canada and Argentina had been vaccinated at weaning with clostridial vaccines and vaccines for bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis, and parainfluenza 3 (some bulls also received *Mannheimia* and *Histophilus somni* vaccines). In all groups, there were no unexpected health issues except in Group 1 in Argentina. In that group, there was a severe outbreak of respiratory disease with high morbidity and 6% mortality, attributed to bovine respiratory syncytial virus (BRSV). In the following year, bulls at this location were vaccinated for BRSV, preventing problems with this disease.

Ultrasonographic examinations in some groups of bulls in western Canada were done monthly from 3 to 15 months of age. In other groups in Canada and Argentina, ultrasonography was done after weaning at 5–8 months of age and repeated at 12–14 months of age in Canada and 18–20 months of age in Argentina. Testis assessment was done by orienting the transducer vertically on the scrotum and moving it 180° around the testis (both dorsal and ventral portions). Testes were scored for fibrosis from none to very severe, based on number and size of fibrotic lesions (Figures 13.1 and 13.2).

Immunohistochemistry was performed on testis tissues of 10 bulls from the group affected by BRSV. Only one of these bulls had fibrotic lesions in testes. Testis tissue was submitted to a diagnostic laboratory for immunohistochemistry for BVDV and BRSV.

Fibrotic lesions scored as slight or mild were very common (31.3%) in testes of a group of bulls raised under intensive rearing conditions in western Canada. Only 2.5% had moderate scores and none had severe scores for fibrosis. In the more extensive management in Argentina, in a group not affected by respiratory disease associated with BRSV, fibrotic lesions were also mainly slight or mild, but incidence was higher (48.6%). In that group, 1.9% were scored moderate and 0.95% severe. In the Argentine group affected by BRSV, fibrotic lesion scores were higher and more frequent. Scores for slight to mild, moderate, and severe to very severe were 58.3, 17.1, and 5.7%, respectively.

Fibrotic lesions appeared as early as 5–6 months of age and number of cases continued to increase until at least 12–14 months. Lesion severity increased in some cases during this period, although it appears that lesion development occurred during a finite interval. There were indications that during fibrosis formation, spermatogenesis was adversely affected; however, presence of a large number of fibrotic lesions that may occupy as much as approximately half of testis parenchyma did not preclude

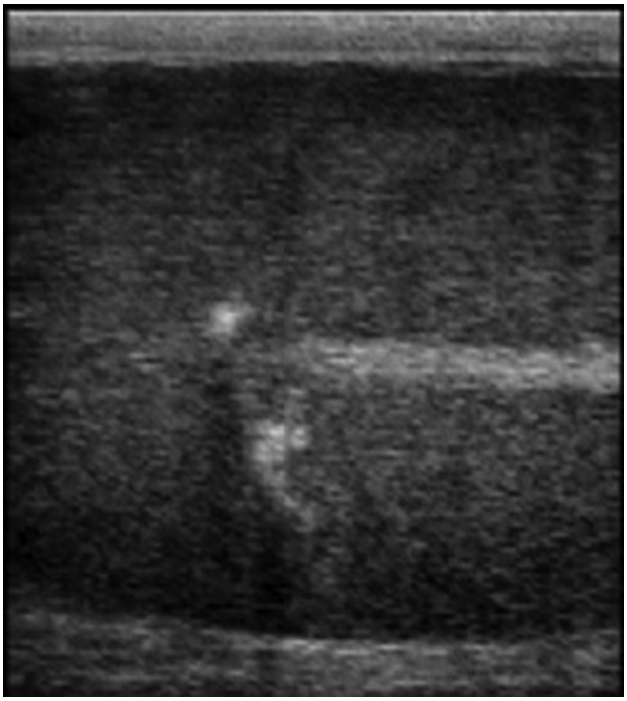


Figure 13.1 An ultrasonogram of testis scored as having mild fibrosis.

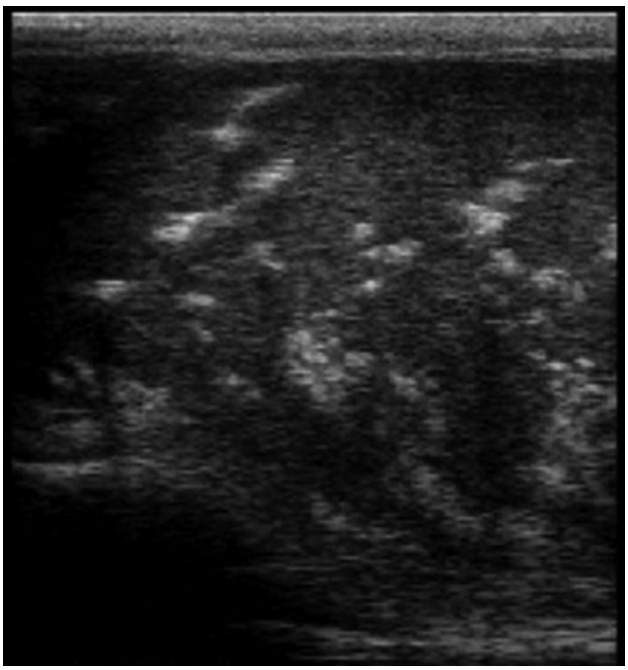


Figure 13.2 An ultrasonogram of testis scored as having very severe fibrosis.

production of a high percentage of morphologically normal sperm. In bulls with no, mild, moderate, or severe testis fibrosis, median percentages of normal sperm were 88, 87, 86, and 90% respectively. Lesion prevalence was not influenced by breed or testis size.

Histologically, lesions consisted of masses of fibrous tissue with fine fibrillation, or heavy bundles of wavy tissue seminiferous tubules. Usually there were fewer germinal cells in seminiferous tubules within masses of fibrous tissue. In some tubules, germinal cells and Sertoli cells were missing entirely and hyaline material was present in the lumen. Similar abnormalities were evident in seminiferous tubules adjacent to fibrous tissue masses; however, completely normal tubules were often beside or very close to fibrous tissue masses. Inflammatory cells were consistently absent. Therefore we inferred that neither the insult that caused fibrosis nor proximity of fibrous tissue to seminiferous tubules affected function of adjacent tubules.

With real-time ultrasonography, many lesions appeared to extend from the rete testis toward the periphery of the testis and appeared to involve the entirety of one or more tubules at specific sites within the testis. This pattern was apparent in both gross sections (Figure 13.3) and in ultrasonograms (Figure 13.4). It appeared that entire tubules may be destroyed with replacement by fibrotic tissue. In other cases, there were isolated lesions in central or peripheral parts of testis parenchyma without extension to rete testis, implying tubular destruction occurred only at a specific site.

The cause of the testicular fibrosis is uncertain. Potential causes are disrupted development of embryonic tubule-to-rete connections, an infectious disease process affecting tubule health and development, abnormal testes thermoregulation, and trauma.

Developmental changes of testes may be involved in development of fibrotic lesions. Tubularization of seminiferous cords within the testis tissue begins at ~4 months of age and is complete in nearly all bulls by 6 months [35].



Figure 13.3 Cross-section of a bull testis with fibrotic lesions that appeared to radiate from rete testis toward the periphery, suggesting individual tubules were destroyed and replaced by scar tissue.

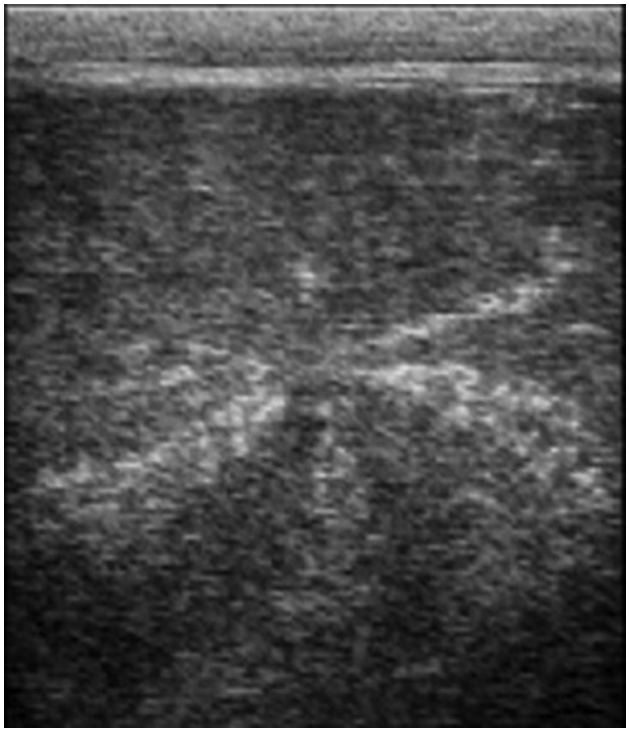


Figure 13.4 Ultrasonogram of a testis with fibrotic lesions radiating from the rete testis toward the periphery.

Sperm release into tubules starts as early as 8 months and occurs in most bulls by 10 months. Therefore tubule impaction with sperm and tubule rupture with escape of sperm into the peritubular space of the testis tissue would not be expected to occur much before 8 months. Because sperm are antigenically foreign, a tissue reaction against escaped sperm could cause fibrosis. Impaction of seminiferous tubules might be due to congenital failure to develop a tubular connection to the rete tubules. Tubule rupture and a tissue reaction against sperm should not occur until testes produce sperm; as production increases, testes lesions would be expected to increase in number and severity.

Bacterial and viral infections are common in early postweaning period when maternal immunity has waned and animals of multiple origins intermingle [36]. Thus an infectious process, as a cause of fibrotic lesions, would fit the time period when testis lesions appear in young bulls. Although bacterial or viral infections would be expected to cause testicular swelling and pain, perhaps inflammation is mild and not apparent. Infectious agents could cause inflammation of arterioles and capillaries in the testes, leading to local tissue necrosis and formation of foci of fibrosis [10]. Viral infections may also target specific cells within seminiferous tubules, e.g. Sertoli cells or individual germinal cells, resulting in tubule damage and fibrous tissue infiltration [37, 38].

Two common viral diseases of cattle, bovine herpesvirus (BHV)-1 and BVDV, have been investigated for their role in male reproductive infections. Both viruses have been isolated from semen [39, 40] and BHV-1 has been associated with degenerative changes in the seminiferous epithelium, perhaps due to illness and fever [41]; however, there are no reports that associate BHV-1 or BVDV with lesions in bovine testes.

The association of a greater prevalence of testis fibrosis with occurrence of a severe outbreak of BRSV respiratory disease in the Group 1 bulls in Argentina, combined with a lesser prevalence of testis lesions in Group 2 (vaccinated against BRSV), suggests that BRSV may be involved in etiology of testis fibrosis. However, immunohistochemistry of testis tissues from 10 bulls that were culled and slaughtered from Group 1 failed to detect BRSV antigen. Failure to find evidence of BRSV antigen may indicate that this virus does not multiply in testis tissue or it might be due to the lack of fibrotic lesions in 9 of 10 of the testes from which samples were submitted and because samples were obtained ~12 months after BRSV incident.

Viral agents multiply within the testes of several species. For example, mature male domestic European rabbits were infected with an attenuated strain of myxoma virus. The animals developed symptoms of myxomatosis 7–10 days after infection and had high viral titers in testes. After 20–30 days, testes were 50% of normal size and affected animals had interstitial orchitis and epididymitis [42]. Protein and genomic studies of a porcine paramyxovirus had a close relationship to human mumps virus [43, 44], known to multiply in human testis, causing orchitis in postpubertal men [45]. Furthermore, when 9-month-old boars were experimentally infected with porcine paramyxovirus, histopathological epididymal alterations and testicular atrophy associated with degeneration of seminiferous tubules occurred [38]. In another study, colostrum-deprived pigs were inoculated with porcine circovirus type 2 alone, porcine parvovirus alone, or with both viruses. All pigs that received both viruses became ill; at necropsy (21–26 days after infection), many had hepatomegaly and enlarged kidneys, with granulomatous lesions apparent in many tissues, including testis [46].

Obesity is associated with testicular degeneration and scrotal insulation has been used to produce testicular degeneration; however, scrotal insulation did not induce fibrotic lesions [4]. In one study, deficient, normal, and excessive dietary intakes did not affect prevalence of fibrotic lesions. However, with prolonged abnormal thermoregulation, especially when semen quantity and quality are very severely reduced, with loss of testis tone and size and with severe seminiferous tubular degeneration, scar tissue infiltration and testis fibrosis are plausible.

Trauma to the testes has also been proposed as a cause of testis fibrosis [10]. Trauma caused by a blow to the testes from kicking or butting could occur at any age, but may be

more frequent during crowding in pens or handling in chute systems. Therefore appearance of fibrosis in conjunction with postweaning penning and feeding could be considered in the etiology of testis fibrosis. However, one of the authors observed a bull receive an acute blow to the testes with a metal pipe. Ultrasonography the following day and several times over the course of a month did not reveal any damage to the testes. Perhaps testis trauma, although common in penned bulls, does not cause fibrotic lesions.

Interestingly, fibrotic lesions in the testes were not associated with poor semen quality. Even bulls with very severe fibrosis produced semen with up to 94% morphologically normal sperm. Therefore presence of relatively large amounts of scar tissue within testis parenchyma did not prevent remaining unaffected parenchyma from producing normal sperm. Although large amounts of scar tissue would be expected to reduce sperm production, this has apparently not been reported.

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14

Vesicular Adenitis

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The vesicular glands are paired glands located on the floor of the cranial pelvic cavity situated laterally and craniodorsal to the prostate and the origin of the paired ampullae. They have also been described as lateral to the ampullae and dorsal to the neck of the bladder. The size of the vesicles is age related. These glands have a lobar structure and each is normally 2–4 cm wide and 10–15 cm long (Figure 14.1). As bulls age, non-infected glands may have considerable variation in size. The vesicular glands are easily palpated per rectum; hence an appreciation of the normal variation is important. As the major accessory sex glands in the bull, infection and inflammation of the vesicular glands has a significant impact on bull fertility. The glands secrete a clear fluid containing nutrients and buffers, which is discharged immediately before and during ejaculation through ducts that open into the urethra adjacent to the colliculus seminalis [1].

The overall prevalence of vesicular adenitis has been reported to be between 1 and 10%, with the highest incidence occurring in yearling and peripubertal bulls and bulls over nine years old [2]. The prevalence is also higher in bulls that are intensively housed and/or fed high energy diets [3]. The actual prevalence of vesicular adenitis in premortem bulls is difficult to establish due to the variance in diagnostic criteria.

Multiple microorganisms have been associated with vesicular adenitis. These include bacteria, chlamydia, mycoplasma, and ureaplasma [2, 3]. Bacteria are considered the most common cause with the most common isolates, including *Trueperella pyogenes*, *Histophilus somni*, and *Brucella abortus* (in areas where brucellosis has not been eradicated). Multiple other pathogens have been isolated from infected vesicular glands [4]. Although the precise mechanism and route of infection is not known, suggested routes of infection include ascending, descending, direct invasion from local sources, and hematogenous routes [2, 4, 5]. Association with congenital defects, particularly involving the development of the colliculus seminalis, as well as naval abscesses in calves have been suggested to predispose bulls to vesicular adenitis [6]. Congenital defects leading to retrograde ejaculation or urine reflux can be a source of infection or sterile inflammation [7]. High energy diets that predispose young bulls to rumen acidosis and rumenitis with subsequent bacteremia may lead to hematogenous vesicular gland infection [2, 3, 7, 8].

Although most bulls diagnosed with vesicular adenitis are found during routine breeding soundness examinations without any history suggestive of vesicular adenitis, occasionally bulls will present with a history of subfertility [3]. Rarely, these bulls may present with acute signs mimicking peritonitis, gastritis, tenesmus, or hindlimb lameness [4].

Physical examination findings, with the exception of those identified through transrectal palpation, are usually normal. Transrectal palpation can reveal a range of findings. Some bulls with vesicular adenitis have no palpable abnormalities with either vesicular gland. Abnormal findings can include any or all of the following: loss of symmetry, bilateral enlargement, loss of lobulation, increase in firmness, heat, and pain (Figure 14.2) [2, 3]. In chronically infected bulls, fibrin and adhesions may be palpable [2, 4].

Bulls suspected of vesicular adenitis should have a thorough examination of the entire reproductive tract. These physical examination findings should be corroborated with semen evaluation. Large numbers of leukocytes with or without flocculi of pus can be found without palpable evidence of vesicular adenitis. Conversely, bulls with palpable signs of vesicular adenitis may have no evidence of infection during semen evaluation. Bulls with transrectal signs of vesicular adenitis but no leukocytes in semen should be further massaged and recollected to ensure

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Figure 14.1 Normal vesicular glands. *Source:* Courtesy Maarten Drost VISGAR.



Figure 14.2 Vesicular adenitis with enlargement and loss of lobulation. *Source:* Courtesy Maarten Drost, VISGAR.

proper evaluation and classification of the presence of disease [2–4, 7]. Transrectal ultrasonography can be revealing in subclinical cases. The normal vesicular gland has homogeneously echogenic lobes with small anechoic vesicles and a distinct anechoic collecting duct all surrounded by a distinct capsule. Vesicular adenitis leads to varying degrees of enlargement, loss of lobular structure, thickened walls, and increased echogenicity with fluid-filled cavities in the gland [9].

Due to likely contamination of samples with environmental microorganisms and normal flora, Parsonson et al. described a technique to obtain vesicular gland secretions for microbiologic examination [10]. After clipping preputial hair, the penis is extended by transrectal massage of the urethralis muscle. The glans penis is restrained by grasping it manually with sterile gauze sponges and wearing sterile gloves. After washing and disinfecting the end of the penis, the urethra is irrigated with sterile saline utilizing a sterile teat cannula. A 25- to 30-cm sterile Silastic tube is passed up the urethra to a point leaving 2.5–5 cm protruding from the penis. The vesicular glands are then massaged and the secretions are collected into a sterile container.

Several treatment modalities for vesicular adenitis have been utilized. These include multiple systemic antimicrobials given at several different dosages and frequencies. Intraglandular antibiotics and intraglandular chemical ablation have shown some success. Surgical removal of chronically infected glands has been described.

Many systemic, parenteral antibiotics have been used in the past to attempt treatment of vesicular adenitis. Since spontaneous recovery commonly occurs in younger bulls, evaluation of treatment methods is complicated [4]. Earlier antimicrobials utilized include penicillin, oxytetracycline, chloramphenicol (no longer legal), sulfamethazine, florfenicol, and cetiofur [2]. These medications all produced poor treatment success rates, even though the bacterial isolates from the vesicular glands are usually sensitive to most antibiotics [2, 11]. Success was improved when increased dosages of these agents was used. Unfortunately, even at twice the recommended dose, these antibiotics often do not reach inhibitory concentrations in vesicular gland tissue [2]. Antibiotics that are highly lipid soluble and low protein binding possess a pH higher than vesicular gland fluid along with a favorable pKa, and should be chosen [4, 12]. Due to the varying clinical signs of vesicular adenitis, establishing whether treatment is successful can be difficult. Treatment can be considered successful when the ejaculate contains less than one neutrophil per five high-powered fields (1000×) [4].

More recently, tilmicosin and tulathromycin have shown efficacy for treatment of vesicular adenitis [13]. The author has treated multiple older bulls successfully using subcutaneous tulathromycin. There is evidence these antimicrobials accumulate in macrophages and neutrophils, which carry the antibiotics into sites of infection to be released slowly [14, 15]. Recovery rates were higher for bulls treated with tulathromycin

than tilmicosin in all age groups; thus tulathromycin at this time appears to be the drug of choice [2].

Intraglandular injection of antimicrobials or chemical ablation agents is another option for treatment of refractory vesicular adenitis. Multiple antimicrobial agents have been utilized in this technique, all with varying results. Success has been achieved in some cases utilizing a single intraglandular injection of ceftiofur or penicillin. In one study, bulls were treated with intraglandular penicillin or intraglandular ceftiofur. Bulls that did not recover were treated with whichever antibiotic was not used initially. Approximately half of the bulls receiving the second treatment responded. Bulls that did not recover after intraglandular treatment with ceftiofur and penicillin recovered after three treatments of tilmicosin [13].

Chemical ablation of chronically infected vesicular glands utilizing 4% formalin has been described [16]. The success of parenteral tulathromycin as an initial treatment has substantially reduced the usage of chemical ablation in the author's practice. Resolution of vesicular adenitis after intraglandular formalin ablation approached 100% when utilized by the author. The procedure requires the bull to be restrained in a chute with epidural anesthesia. After clipping and prepping an area lateral to the anus, a 14-gauge needle is placed through the skin directly caudal to the affected vesicular gland (Figure 14.3). The affected gland is grasped transrectally to stabilize it for injection and a 30-cm, 18-gauge needle is introduced through the 14-gauge needle (Figure 14.4). This needle is advanced pararectally to achieve penetration of the affected vesicular gland. When sure the needle is within the gland, inject 10–15 ml of saline to ensure proper needle placement. Gland enlargement verifies proper needle placement. At this point, inject 4% formalin to a volume where the gland is enlarged with a smooth surface (Figure 14.5). In the author's experience, this will be approximately 50 ml. After injection, the bull should be treated with flunixin meglumine for three days, as most bulls show signs of abdominal discomfort. The proper authorities should be consulted regarding current legal use and withdrawal time prior to the extra label use of formalin for chemical ablation.



Figure 14.3 Placement of a 14-gauge needle through the skin. *Source:* Courtesy Josh Thompson.



Figure 14.4 Passing a 30-cm, 18-gauge needle through the 14-gauge needle into the vesicular gland. *Source:* Courtesy Josh Thompson.



Figure 14.5 After removing the stylus from the needle, the proper solution is injected into the vesicular gland. *Source:* Courtesy Josh Thompson.

Surgical removal of the vesicular glands may be considered in bulls that do not spontaneously recover or do not respond to antimicrobial treatment or ablation. A subrectal approach has been described that provides better visualization of the vesicular glands and improved postsurgical fertility than traditional pararectal approaches. None of the described techniques for surgical gland removal has provided uniform success in returning a bull to function [1].

Any specific plan for prevention of vesicular adenitis is limited by the lack of a definitive pathogenesis. Proper herd health and husbandry may play an important role in reducing the prevalence of vesicular adenitis. Proper

vaccination and deworming help support the overall health and general disease resistance of bulls. Proper nutrition and bunk management to reduce the incidence of rumen acidosis may reduce the possibility of hematogenous bacteria reaching the vesicular gland. Monitoring the genetic lines of bulls with vesicular adenitis may reveal a genetic basis for the disease. Although early detection, with or without medical intervention, has not shown a significant reduction in the incidence of clinical vesicular adenitis [17], herds with a high prevalence of adenitis may benefit from early transrectal palpation and treatment [2].

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15

Inability to Breed Due to Injury or Abnormality of the External Genitalia of Bulls

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Introduction

The inability to sire offspring is referred to as impotence and may result from abnormalities in sperm production and/or inability to copulate. Inability to copulate (impotentia coeundi) is further divided into inability to perform the copulatory act due to physical limitations and inability to copulate due to erection failure (impotentia erigendi). This chapter reviews important causes of impotence due to abnormalities of the genitalia of the bull. Abnormal spermatogenesis is covered in Chapters 3 and 5.

Impact of Impotence

Natural service typically utilizes bull to cow ratios of 1 : 15 to 1 : 50 [1]. The inability of the bull to breed in a single sire unit will always have a devastating effect on reproductive efficiency. Similarly, when a socially dominant bull in a multisire unit is unable to complete the copulatory act, the decrease in breeding efficiency may not be masked by the presence of additional subservient bulls. Although artificial insemination eliminates the requirement for coitus, most semen processed for the artificial insemination industry is collected by methods that mimic the copulatory act utilizing an artificial vagina (AV) as the bull mounts a teaser or phantom. While some impediments to the copulatory act may be overcome by the collection of semen using electroejaculation, others may not be easily managed.

Physiology of Erection in the Ruminant

The unique and interesting features contributing to extension and erection of the penis of the bull are well described [2, 3]. A brief summary of the process follows.

The fibroelastic ruminant penis is encased in the rigid tunica albuginea which encloses the erectile tissues of the corpus cavernosum penis (CCP). In the non-erect state the penis is maintained within the prepuce and sheath by the traction of the retractor penis muscles, which insert on the ventral surface of the mid-shaft of the penis at the distal bend of the sigmoid flexure. The proximal terminus of the CCP is the bulb of the penis, which divides laterally into two crura which are covered by the ischiocavernosus muscles at they attach to the bony pelvis at the ischiatic arch.

At the initiation of sexual arousal, the retractor penis muscles relax in synchrony with increased blood flow from the deep arteries of the penis into the crura, and the CCP fills with blood. The loss of muscle tension from the retractor muscles coupled with complete filling of the CCP initiates partial straightening of the sigmoid flexure. Contractions of the ischiocavernosus muscles compress the blood-filled penile crura against the pelvis and occlude all vascular flow to and from the CCP, resulting in a closed hydraulic system. Further rhythmic contractions of the ischiocavernosus muscles increase the pressure of the blood contained within the crura and the CCP to produce the force necessary for full erection and extension of the penis. The pressure generated in the CCP far exceeds the arterial pressure of the circulatory

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

system, reaching 14 000 mmHg (1.87 MPa) or higher at the time of peak erection in the bull [4].

Coitus

The breeding act requires orchestration of interrelated simple and complex events. The bull must identify the female in estrus, approach, extend the penis, mount appropriately, achieve full erection, adjust his stance and posture, blindly search for the vulva with the penis, achieve intromission, make the breeding lunge, ejaculate, and dismount. The physical requirements are obvious and structural soundness is a necessary component of the successful breeding act.

In cattle the coital act occurs rapidly, with time from mounting to intromission and ejaculation generally less than 10 seconds and the time from intromission to ejaculation 1–2 seconds [5, 6]. Some non-productive false mounts are expected, but excessive numbers may be associated with pathology of the axial or appendicular skeleton, problems with the erection process, impediments to penile protrusion and extension, or lack of innervation of the glans penis.

Following a successful breeding attempt the bull has a brief refractory period, the length of which varies among bulls and is likely affected by age, physical condition, and the presence of other bulls and females exhibiting estrus. For young physically sound bulls the refractory period may be only a few minutes and most bulls can be expected to copulate multiple times per day.

Abnormalities of the Prepuce

The prepuce is an invagination of abdominal skin that contains the free portion of the non-erect penis and covers the body of the penis behind the glans. The preputial orifice is located caudal to the umbilicus and is surrounded by a tuft of long hair that serves to divert urine away from the skin surrounding the orifice following micturition.

The penile attachment of this invagination of skin occurs caudal to the glans penis and in the non-aroused state penile retraction results in the presence of a fornix in the preputial cavity adjacent to the free portion of the penis. When erect, the free portion of the penis extends beyond the preputial orifice and the hairless reflection of skin within the preputial cavity extends with the penis and covers the shaft. The subcutaneous tissues of the prepuce must be freely moveable to allow extension and retraction of the penis and the preputial reflection. The specialized connective surrounding the tunica albuginea are referred to as the elastic layers of the prepuce.

Congenital and Developmental Anomalies

Congenital Preputial Stenosis

Congenital preputial stenosis will prevent extension of the penis, but stenosis secondary to injury or laceration of the preputial tissues is more common.

Incomplete Separation of the Penile and Preputial Epithelium

Prior to puberty, the epithelium of the free portion of the juvenile penis and the integumentary epithelium of the penile portion of internal lamina of the prepuce are joined by an interdigitating attachment [7–9]. Exposure to the trophic effects of androgens as bulls approach puberty leads to an increase in penile size and to development of the sigmoid flexure characteristic of ruminants. Simultaneous with the increase in penile size, the epithelial attachments between the preputial skin and the epithelial surface of the free portion of the penis weaken and begin separating. Separation may be facilitated by mounting activity and early attempts at erection during sexual role playing by young bulls. Until the separation is complete at 8–11 months of age, extension of the penis is not possible [7, 8, 10].

Normal separation is sometimes delayed and may result in an inability to fully extend the penis at the time of a breeding soundness examination or during an observed breeding attempt (Figure 15.1a). The attachment will often spontaneously regress in affected bulls if allowed adequate time to mature, but assisted separation by gentle application of manual traction to the penis is sometimes advocated in cases where partial separation has already occurred (Figure 15.1b and c).

Young bulls at times tear the epithelial attachment prematurely, and hemorrhage into the space between the penile and preputial epithelium results in a localized hematoma. Such injuries may go unnoticed and are often self-limiting [10]. If bacteria gain access to the damaged tissues and associated hematoma, abscess formation and fibrosis may compromise the ability to extend the penis. Case management should emphasize isolation from other animals to discourage sexual role-playing during the healing period, accompanied by broad-spectrum antibiotic treatment to minimize complications. Drainage of an abscess in this location is difficult and must be done with care to minimize loss of the elastic layers of the prepuce.

Persistent Frenulum

The frenulum is a thin band of tissue found in juvenile bulls that extends from the midline of the ventrum of the tip of the penis to the attachment of the preputial epithelium near the base of the free portion. Like the epithelial connection between the penis and prepuce, this band of tissue normally separates under the influence of androgen

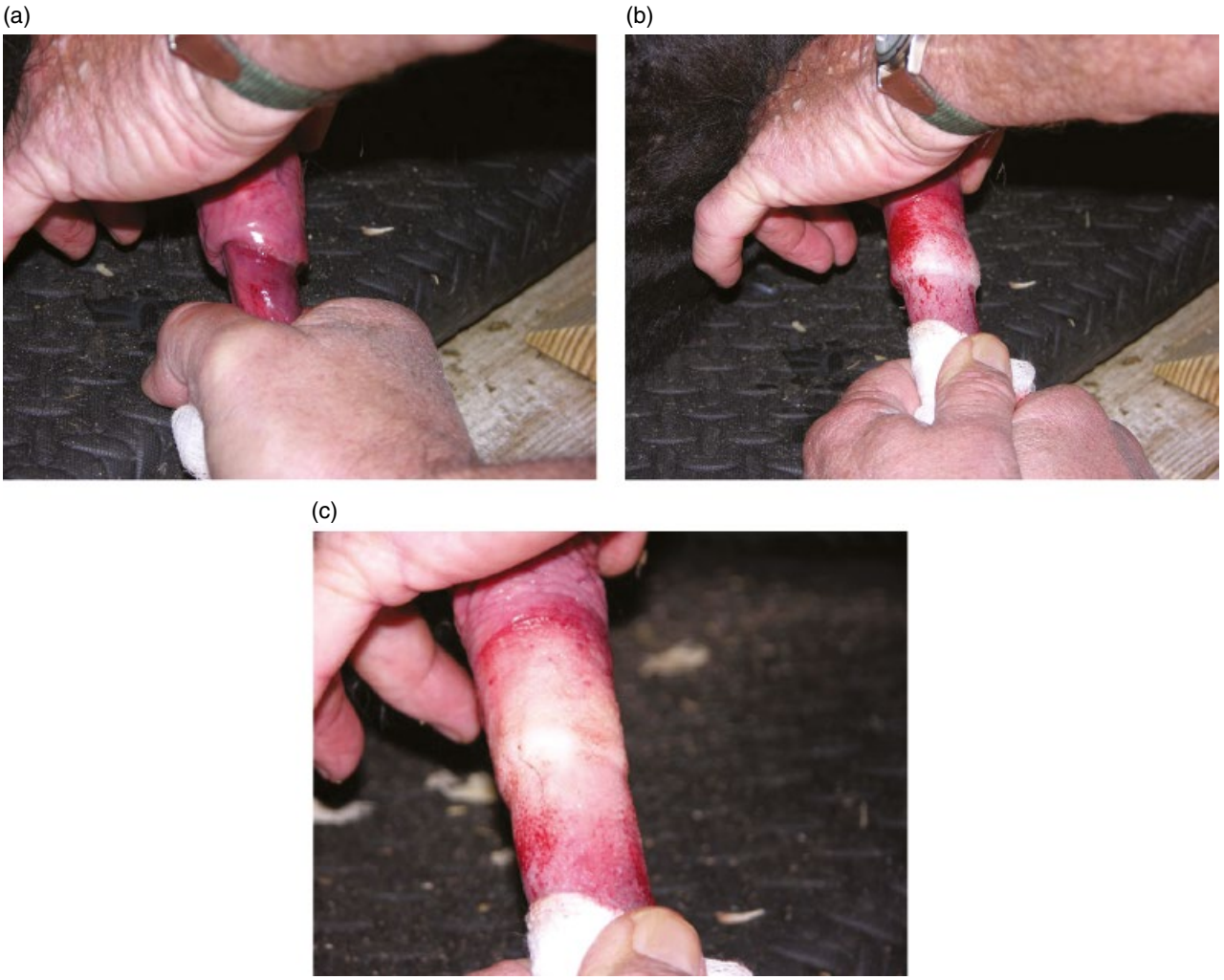


Figure 15.1 (a) Failure of separation of the epithelium of the free portion of the penis and the penile portion of the prepuce. (b) Note the adherence between the preputial and penile skin. Application of gentle traction to the penis to facilitate adherence breakdown. (c) Penile and preputial epithelium following breakdown of the epithelial adherence. *Source:* Courtesy of Robert L. Carson and Dwight Wolfe.

exposure as the bull matures. Unlike the epithelial tissues joining the penis and prepuce, the frenulum often contains a large vein. When the process of normal separation fails, the frenulum may persist after breakdown of the other epithelial attachments and result in a band of epithelium and connective tissue joining the tip of the penis with the preputial epithelium at the junction of the prepuce and free portion of the penis (Figure 15.2). A persistent frenulum results in a sharp ventral bend of the distal penis as it becomes erect. Persistent frenulum is often detected in young bulls during a routine preservice breeding soundness examination [11], but occasionally is not detected until bulls attempt natural service.

The cause or causes of failure of separation of the preputial epithelium and/or frenulum are unknown. An unproven genetic association is suspected [12, 13]. Surgical transection of the frenulum is curative [13, 14], but limiting affected animals to use as terminal sires is recommended.

Injuries to the Prepuce

Bulls sometimes sustain injuries to the prepuce severe enough to interfere with breeding. Bulls from breeds with substantial *Bos indicus* influence have a pendulous sheath, excessive preputial skin, and a large preputial orifice, and bulls of this phenotype are predisposed to preputial trauma at the time of breeding. While *Bos taurus* breeds have less redundant skin in the area of the prepuce and are less likely to sustain breeding-associated preputial trauma, preputial injury at the time of the ejaculatory lunge may occur in any breed.

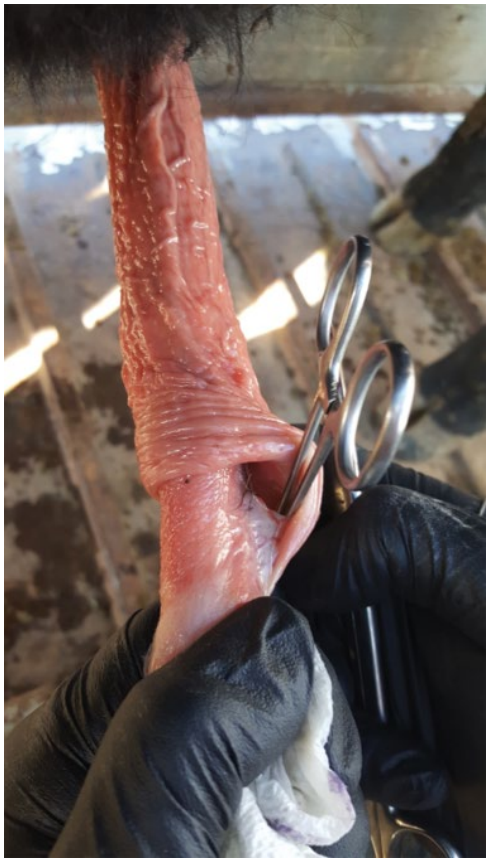


Figure 15.2 Persistent frenulum. The presence of the persistent frenulum was noted at the time of a post-purchase breeding soundness examination. A satisfactory pre-purchase "semen test" had been provided at the time of sale, emphasizing the importance of examination of the external genitalia during a bull breeding soundness examination. *Source:* Image courtesy of Dr. Clint Hilt.

Preputial Laceration

Bulls with a pendulous sheath and excessive preputial skin may traumatize the preputial tissues independent of the breeding act, but most serious preputial injuries occur at the time of the ejaculatory lunge. As the free portion of the penis enters the vagina during coitus, preputial skin slides caudally, up the shaft of the penis, toward the abdomen of the bull, and folds of redundant skin gather at the preputial orifice. This "bunching" of preputial skin usually occurs without incident, but when preputial tissue is inadvertently trapped between the abdomen of the bull and the bony pelvis of the female at the time of intromission, compressive forces generated at the time of the ejaculatory lunge can injure the entrapped tissues. With mild injury, the preputial epithelium remains intact and the accumulation of edema in the damaged tissues results in an uncomplicated preputial prolapse. More serious injuries result when compression of the entrapped prepuce disrupts the epithelium, with subsequent exposure and damage of the underlying elastic tissues. Commonly referred to as preputial laceration, the injury is in reality the result of bursting of preputial tissues in response to compressive force [15].

Preputial lacerations initiated during the breeding act are predictably located on the ventrum of the prepuce, with the initial disruption of the preputial tissues oriented longitudinally, parallel to the long axis of the bull's body. Following the injury, the disrupted tissues at the site of the injury become oriented transversely as the penis is retracted and the damaged preputial tissues are drawn toward the preputial orifice. As a result, the defect becomes oriented transversely (Figure 15.3). When the damaged preputial tissues cannot be retracted through the preputial orifice, the transverse orientation of the lacerated tissues results in shortening of the caudal aspect of the exposed prepuce and a characteristic "elephant trunk" appearance of the prolapsed tissues (Figure 15.4).

Trauma and disruption of the preputial epithelium and underlying elastic tissues is accompanied by inflammation and edema and the open wound inevitably becomes septic. As dependent edema accumulates, the prolapsed tissue increases in size and weight, and traction on the prepuce results in greater amounts of preputial tissue becoming exposed. Additional trauma, mutilation, and desiccation of the unprotected preputial tissues occur and wound contracture at the site of the injury distorts the tissues as the reoriented wound undergoes fibrosis and cicatrix formation (Figure 15.5).

Preputial retractor muscles serve to elevate the prepuce and this elevation can minimize edema formation in damaged tissues. Many polled bulls lack retractor prepuce muscles and preputial prolapse following laceration in naturally



Figure 15.3 Preputial laceration in a breeding bull. The laceration assumed a transverse orientation as the preputial tissues were retracted toward the preputial orifice.



Figure 15.4 Preputial laceration with prolapse of the damaged preputial tissues. Reorientation of the wound results in the elephant trunk appearance of the prolapsed preputial tissue.



Figure 15.5 Wound contracture and fibrosis at the site of a preputial laceration. *Source:* Courtesy of Richard Hopper.

hornless bulls tends to become more severe than similar injuries in horned animals [16].

Wolfe and Carson constructed a four-point classification scheme that incorporates the severity of the preputial injury to estimate the prognosis for return to function and guide treatment decisions (Table 15.1) [17].

Medical management of preputial laceration and prolapse is aimed at control of tissue sepsis, reduction of edema, and the eventual return of the damaged tissues to the preputial cavity. Application of emollients to prevent desiccation and topical antibiotics should be combined with light bandaging. Careful cleansing and flushing of the wound with dilute antiseptic solutions and debridement of devitalized tissues is necessary. Topical antibiotic therapy is sufficient if wound management is adequate, and systemic antibiotic therapy is not often required.

Table 15.1 Classification of preputial prolapse.

Category	Description	Treatment and prognosis
I	Simple preputial prolapse with slight to moderate edema without laceration, necrosis, or fibrosis	Either conservative or surgical treatment with good prognosis
II	The prolapsed prepuce has moderate to severe edema, may have superficial lacerations or slight necrosis, but has no evidence of fibrosis	Surgery is the usual course of therapy with a good to guarded prognosis
III	There is severe edema of the prolapsed prepuce with deep lacerations, moderate necrosis, and slight fibrosis	Surgery is indicated and the prognosis is guarded
IV	The prolapsed prepuce has been exposed for quite some time and has severe edema, deep lacerations, deep necrosis, fibrosis, and often abscess	Surgery and salvage by slaughter are the only options, and a guarded to poor prognosis follows surgery

Source: Modified from [17], p. 258, © 1998, Wolters Kluwer.

Application of a circumferential bandage to protect the wound, prevent desiccation, and apply mild compression of the damaged tissues is useful. To apply a bandage following cleansing and application of an emollient antibiotic ointment, first place a short length of latex tubing into the preputial orifice and position it with one end in the preputial cavity proximal to the torn epithelial tissues and the other end exiting the preputial orifice to allow urine egress from the prepuce. Then place a piece of clean 5-cm orthopedic stockinette over the exposed preputial tissues and snugly apply an elastic tape bandage over the stockinette, prepuce, and urine egress tube beginning at the distal end of the prolapsed tissue, overlapping the tape as it advances up the prepuce to the preputial orifice, where it can be secured to the haired skin of the sheath (Figure 15.6). Following bandaging, the edematous prepuce may sometimes need to be suspended by application of a bib or sling made of net material or burlap supported by straps encircling the bull's abdomen (Figure 15.7). Frequent bandage changes are necessary, and the wound should be treated locally each time it is exposed. Cold water hosing for 10–15 minutes at each bandage change will reduce edema and remove necrotic debris. With diligent treatment many bulls may be returned to service without surgery [18] but repeat injury is common.

Surgical treatment following preputial laceration can improve outcome [19] and is indicated when the bull's

(a)



(b)



Figure 15.6 Bandaging of the prolapsed preputial tissues following application of an emollient and topical antibiotics. Placement of a urine egress tube to evacuate urine from the prepuce, a light stockinette to protect the exposed tissues (a), and an overlapping elastic tape pressure bandage secured to the preputial hairs and urine egress tube distally and to the skin of the haired sheath proximally (b).



Figure 15.7 Burlap "bib" applied to the bull's abdomen to suspend the edematous preputial tissues. *Source:* Image courtesy of Chance Armstrong.

value and remaining breeding life justify the expense [19, 20]. Surgery must always be preceded by preoperative wound management. Excellent descriptions of the surgical options appear elsewhere in this book (see Chapter 19).

Retropreputial Abscess

Preputial injury and laceration are not limited to *B. indicus* influenced breeds. In *B. taurus* bulls, preputial injury may occur at the time of breeding in a manner identical to that described for *B. indicus* bulls, but the outcome is often altered by phenotype. *B. taurus* breeds are more likely to retract all the damaged tissues into the preputial cavity following injury and as a result the wound is less likely to be noticed early. The visible preputial swelling may be confined to a well-defined area adjacent to the bull's sheath, or may be more diffuse and occasionally extend from the preputial orifice caudally toward the scrotum. Because the compromised elastic tissues within the preputial cavity are contaminated with bacteria, cellulitis and phlegmon develop rapidly, often progressing to abscess formation.

Retropreputial abscess formation is more likely in bulls of *B. taurus* than *B. indicus* influenced breeds due to lack of redundant skin and generally tighter sheath conformation. Affected bulls present with an obvious swelling visible through the overlying skin of the sheath that may be accompanied by the presence of pus or blood at the preputial orifice. Diagnosis is based on physical examination and palpation, sometimes augmented by ultrasound imaging of the tissues. The differential diagnosis for preputial inflammation with visible disruption of the normal contour of the sheath must include the enlargement of the elastic tissues seen following rupture of the tunica albuginea of the penis. In contrast to the lesion seen with rupture of the tunica albuginea, retro preputial abscesses are usually non-symmetrical and located distal to the sigmoid flexure nearer the level of the preputial fornix (Figure 15.8). Retropreputial abscess formation is associated with poor prognosis for future breeding. Destruction and impairment of the elastic tissues frequently result in adhesion formation within the elastic tissues of the prepuce and the overlying skin, or in compromise of the diameter of the preputial lumen, either of which may prevent extension of the penis [17].



Figure 15.8 Retropreputial abscess following preputial laceration in a young bull. Note location of the swollen tissues in the distal sheath.

Therapy for retropreputial cellulitis, phlegmon, and abscessation relies on systemic antibiotic administration and local wound management. Daily flushing of the preputial tissues with dilute antiseptic solutions and cold water hosing of the sheath aid in resolution of cellulitis. Drainage of a retropreputial abscess into the preputial lumen at the site of the original injury may facilitate recovery but is difficult to accomplish. No attempt should be made to drain a retropreputial abscess through the overlying skin of the sheath as inflammation and sepsis of the underlying elastic tissues are inevitable and subsequent formation of peripenile adhesions will decrease the chance of a successful outcome [17, 18]. Even with aggressive therapy the prognosis is guarded to poor and many affected bulls never return to service [17].

Phimosis

Phimosis, the inability to extend the penis, effectively prevents the bull from breeding and may be diagnosed at the time of an observed breeding or by induction of erection with an electroejaculator. Phimosis may be due to stenosis of the preputial opening or lumen, adhesions within the elastic layers of the prepuce and surrounding skin, or occasionally abnormalities of the distal penis including the presence of large penile fibropapillomas.

Stenosis or stricture of the preputial lumen can occur following preputial injury despite appropriate and apparently successful medical or surgical management. Scar tissue replaces damaged elastic tissues, and contracture and cicatrix formation may constrict the preputial lumen and result in preputial stenosis sufficient to prevent extension of the penis (Figure 15.9). If circumferential constriction of the preputial cavity occurs distal to the end of the tip of the non-erect penis, sexual arousal and engorgement of the penis will force the distal portion of the penis down the prepuce until the restriction is encountered and the preputial lamina will then be forced out the preputial orifice without exposure of the free portion of the penis. Strictures sometimes interfere with the evacuation of urine from the preputial cavity. Non-circumferential scar formation at the site of a healed preputial laceration may also compromise the preputial lumen and prevent penile extension.

Extension of the penis requires appropriate function of the elastic tissues and severe damage prevents the gliding action that permits the free portion of the penis and internal lamina of the prepuce to exit the preputial orifice. Formation of adhesions between the penis, the elastic tissues, and overlying skin following trauma may effectively limit the movement of the penis and result in partial or complete failure of extension. The site of the adhesions may sometimes be identified by a visible deformation of the contour of the overlying skin at the time of attempted erection.

Phimosis may also result due to the presence of a penile wart (papilloma) larger than the external opening of the preputial orifice. Incision of the internal lamina of the prepuce to allow extension of the penis and removal of the wart followed by primary closure of the incised preputial tissue can be curative if the value of the bull is sufficient to



Figure 15.9 Phimosis due to circumferential stricture of the preputial cavity following preputial laceration. *Source:* Courtesy of Craig Easley.

justify the expense of surgery (Figure 15.10). Restraint on a tilt table and administration of local analgesia by blocking the dorsal nerves of the penis or administration of an internal pudendal nerve block facilitate this procedure.

Many bulls with phimosis are culled. In cases of preputial stenosis, restoration of the preputial lumen may be accomplished by resection of the compromised tissues and anastomosis of the remaining prepuce if sufficient healthy preputial tissues remain. Similarly, surgical scar revision can be useful for restoring the integrity of the preputial lumen when the amount of healthy preputial skin is insufficient for preputial resection. Both techniques are described in Chapter 19. No treatment is effective if adhesions of the elastic tissues are severe.

Avulsion of the Prepuce

The attachment of the reflection of preputial skin to the free portion of the penis is susceptible to avulsion injury at the time of breeding, but this injury occurs more commonly when semen is collected with an AV. If the AV is inappropriately sized or not properly lubricated, preputial skin will not easily slide down the interior of the latex liner and can be avulsed or torn from its attachment to the penis at the time of the ejaculatory lunge. This injury can often be recognized immediately following service with the AV when blood is seen at the external preputial orifice. Extension of the penis allows visual observation of the avulsion injury, typically located on the ventrum of the penis (Figure 15.11) [21]. Unlike preputial lacerations which occur at the time of breeding, the underlying tissues are not severely traumatized and immediate repair by direct suture of the avulsed tissues is recommended. Prognosis following early detection and repair is good [21]. If not noticed early and repaired immediately, hemorrhage and wound sepsis may complicate management but careful dissection and reattachment of the avulsed tissue can still be successful.

When collecting semen with an AV the person collecting the semen should be appropriately trained, a suitable AV and liner should be selected and properly prepared, and bulls should always be observed closely following collection [21].

Abnormalities of the Penis

Congenital Abnormalities

Congenital abnormalities of the penis occur sporadically and are sometimes associated with other disorders of sexual development.

Hypospadias

Hypospadias, an abnormal opening in the urethra that occurs when the embryonic urethral groove fails to close or closes incompletely anywhere along its length, has been reported in bulls and other species. In some cases the entire length of the penile urethra may be involved and may be easily observed at birth [22]. Cases that involve only the distal urethra may go unobserved until the bull is examined during a breeding soundness examination [23]. Slight caudal relocation of the urethral meatus may not significantly affect fertility.

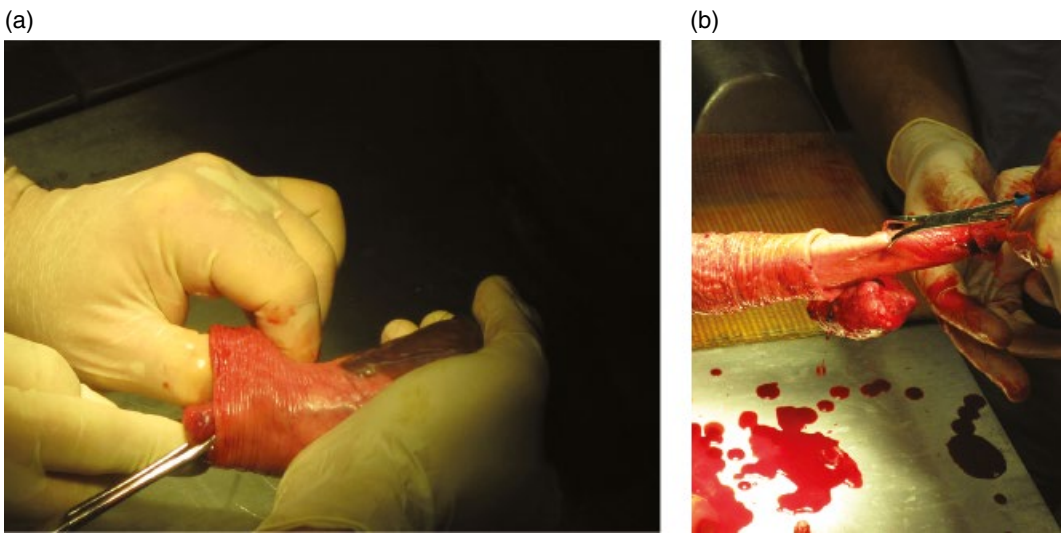


Figure 15.10 Phimosis due to the presence of a penile fibropapilloma larger than the preputial orifice. The tip of the free portion of the penis has been grasped with sponge forceps (a). Following incision of the internal lamina of the prepuce to enlarge the preputial orifice, the penis was extended and the fibropapilloma surgically excised (b).



Figure 15.11 Preputial avulsion. Notice the separation of the attachment of the preputial skin from its attachment at the base of the glans penis. Such injuries most commonly occur during collection of semen in a poorly prepared AV but sometimes, as in this case, occur during natural breeding. *Source:* Image courtesy Jessica Rush.

Congenital Short Penis

Congenital short penis is seen uncommonly and must be differentiated from a partial phimosis secondary to preputial injury or fibrosis of the retractor penis muscles. Some affected bulls may sire calves by natural service during their first or second breeding season, but copulation becomes impossible as they reach mature body size. Diagnosis is made by observation of breeding and measurement of the distance from the preputial orifice to the tip of the extended penis. The etiology of congenital short penis is unknown [24]. Affected bulls are unable to breed naturally and should be culled.

Diphallus

Diphallus, or duplication of the penis, is reported sporadically [25, 26]. Duplication may involve only the glans penis, or the free portion of the penis may be involved.

Congenital Vascular Shunts

Anomalous vascular anastomoses between the peripenile circulation and the erectile tissues of the CCP result in shunts that allow blood from the CCP to exit the erectile tissues and destroy the integrity of the closed hydraulic system necessary for complete erection [27]. Vascular shunts may occur as a congenital anomaly or form following an injury that disrupts the integrity of the tunica albuginea of the penis. Regardless of etiology, when erection is stimulated at a test breeding or with an electroejaculator, partial erection and protrusion of the glans penis is initiated followed by loss of intrapenile pressure and failure to achieve full erection.

Vascular anastomoses between the CCP and extracorporeal vasculature found in the distal free portion of the penis are usually multiple and result from congenital flaws in the integrity of the tunica albuginea [28]. As blood escapes through these distally located shunts at the time of sexual stimulation, "blushing" of the preputial tissues may be observed as blood from the CCP enters the venous circulation beneath the preputial skin. A presumptive diagnosis may be made following observation of a test mating or attempts to induce erection with an electroejaculator. Confirmation requires demonstration of the shunts with radiographic contrast studies in which radiopaque contrast media are injected directly into the CCP as serial radiographs are taken. In the normal penis the contrast media remain within the CCP until eventually exiting at the crura of the penis (Figure 15.12). Visualization of the contrast media within the peripenile vasculature outside the CCP is diagnostic (Figure 15.13). Demonstration of anomalous vessels in the distal portion of the penis may be enhanced by occlusion of the distal peripenile vessels by application of a tourniquet to the penis proximal to the location of the shunt(s) [29]. Unlike acquired vascular shunts of the CCP, congenital shunts are seldom amenable to surgical correction.

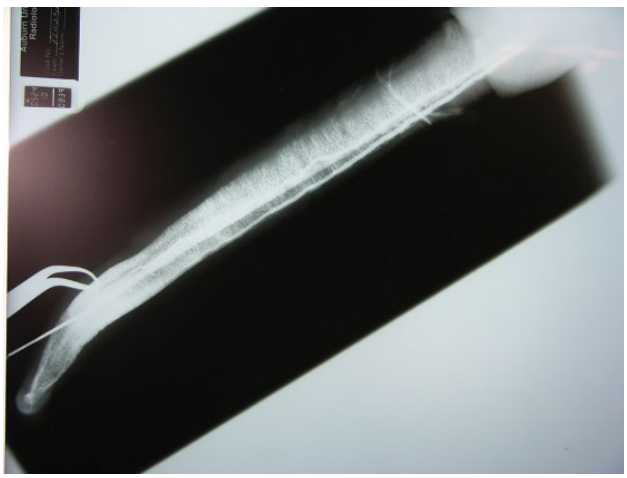


Figure 15.12 Normal cavernosogram. Contrast media injected into the CCP at the level of the free portion of the penis remain within the CCP. The ventral canals and cavernous spaces are clearly outlined.

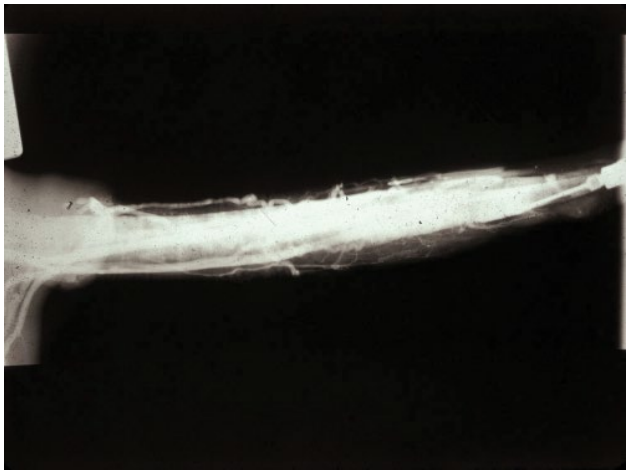


Figure 15.13 Cavernosogram demonstrating multiple shunts from distal cavernous spaces to the peripenile vasculature. *Source:* Courtesy of Robert L. Carson and Dwight Wolfe.

Anatomic and Developmental Abnormalities

At the time of puberty androgens initiate growth of the juvenile penis and initiate separation of the penile and preputial epithelium [7], as discussed in the section "Abnormalities of the Prepuce." The sigmoid flexure develops as the penis enlarges and the retractor penis muscles attached to the proximal flexure of the sigmoid maintain the non-erect penis within the sheath. The rigid tunica albuginea has sufficient strength to effectively contain the pressures generated at the time of erection and gives the erect penis its tubular shape. Uniform filling of the cavernous spaces of the CCP is required for complete erection. The dorsal apical ligament of the penis must elevate the free portion of the erect penis to allow successful intromission.

Penile Deviation

Deviation of the penis results in inability to copulate. Affected bulls may have a history of one or more successful breeding seasons and there is often no history of trauma or penile injury. In the bull the dorsal apical ligament of the penis originates from the tunica albuginea proximal to the free portion of the penis, runs along the dorsum of the free portion beneath the penile skin, and rejoins the tunica albuginea near the distal end of the CCP. The ligament inserts on the distal penis centrally with a broad set of fibers and on the left lateral aspect of the free portion of the penis with a narrower and better defined set of fibers [9, 30]. This ligament gives support to the erect penis and maintains the normal alignment of the penis as the bull positions himself for coitus and utilizes the tip of the penis to search for the vulva. Following intromission and achievement of peak erectile pressure the ligament may "slip" to the left and the distal penis will often assume a corkscrew shape and spiral within the vagina at the time of the ejaculatory lunge [6]. When erection is induced with the artificial stimulus of an electroejaculator, the penis will frequently assume this same spiral orientation, often prior to ejaculation.

Spiral Deviation

Pathologic spiral deviation of the penis occurs when the dorsal apical ligament slips laterally prior to intromission (Figure 15.14a). The role of the dorsal apical ligament in the development of spiral deviation is well described, but the factor or factors leading to the premature occurrence of this otherwise normal phenomenon are poorly understood [9, 30, 31]. Malfunction of the dorsal apical ligament due to a shortening of the ligament or lengthening of the penis as the bull ages was once commonly felt to be the cause but remains unproven. More recent speculation suggests that intravaginal spiral deviation occurs normally in many bulls, associated with peak erectile pressure, and that pathologic spiral formation occurs prematurely in bulls in which peak erectile pressure occurs prior to intromission. In either case, bulls with premature spiral deviation of the penis are unable to complete the copulatory act.

A diagnosis of spiral deviation is suspected based on history and a description of the penis during the breeding attempt, but diagnosis can only be confirmed by an observed test mating. Because the penis of many normal bulls will spiral under the artificial stimulus of electroejaculation, diagnosis following observation of spiraling during stimulation with an electroejaculator is insufficient (Figure 15.14b). Deviation may be intermittent, especially early in the development of the condition, and repeated observations of test matings may be required to confirm the diagnosis.

No medical therapy is available to correct spiral deviation of the penis. Surgical correction of spiral deviation relies on induction of fibrous tissue to strengthen the attachments of the dorsal apical ligament to the penis. The available surgical techniques are described in Chapter 19. Spiral deviation is sometimes seen in association with damage to the dorsal nerves of the penis and careful observation at the time of test mating to rule out penile denervation should precede any attempted surgical correction.

Ventral Deviation

Ventral deviation of the penis is less common than spiral deviation and the etiology is uncertain. The penis assumes a ventral curvature as erection progresses and has been described as a "rainbow" due to the arc formed by the erect penis. Like spiral deviation, ventral deviation is best diagnosed with an observed test mating, but observation under



Figure 15.14 (a) Spiral deviation of the penis demonstrated during a test mating. (b) Spiral deviation of the penis induced with an electroejaculator during semen collection. Spiraling of the penis during electroejaculation is common and should not be confused with the pathologic deviation prior to achieving intromission shown in (a). *Source:* Image courtesy of Clint Hilt.

stimulation with an electroejaculator is useful because, unlike spiral deviation, ventral deviation is not a normal phenomenon. The area of ventral deviation usually originates in the shaft of the penis proximal to the origin of the dorsal apical ligament (Figure 15.15), meaning that surgical correction of ventral deviation with techniques to supplement the ligament are unlikely to be successful. The occasional reports of successful correction by pexy of the dorsal apical ligament are limited to cases in which the deviation is restricted to the free portion of the penis [32] (Figure 15.16).

S-Shaped Deviation

S-shaped deviation of the penis is the least common type of penile deviation. It develops in mature bulls and the serpentine curvature of the penis appears to result from a mismatch of the length of the penis and dorsal apical ligament. The penis may appear excessively long or the apical ligament may have undergone contracture following repeated injury. The dorsal apical ligament prevents the fully erect penis from assuming its normal straight orientation and the bull may be unable to control the penis sufficiently to locate the vulva of the cow. Semen from affected bulls



Figure 15.15 Ventral deviation of the penis demonstrated during stimulation with an electroejaculator. Note that the deviation begins proximal to the origin of the dorsal apical ligament of the penis. Surgical repair has been unsuccessful in such cases. *Source:* Courtesy of Robert L. Carson and Dwight Wolfe.



Figure 15.16 Ventral deviation of the penis demonstrated during a test mating. Note that in this case the deviation begins distal to the origin of the dorsal apical ligament of the penis. Surgical supplementation of the dorsal apical ligament may be attempted in these cases. *Source:* Courtesy of Richard Hopper.

could be collected for use in artificial insemination, but no effective treatment for the condition exists.

Persistent Frenulum

Persistent frenulum has been described under "Abnormalities of the Prepuce." Diagnosis by observation is obvious but may sometimes be confused with phimosis in bulls with excessive redundant preputial skin when the ventral bending of the prepuce prevents extension of the glans beyond the preputial orifice (Robert L. Carson, personal communication).

Penile Injury

Urethral Fistula

A urethral fistula may develop following laceration of the urethra, constrictive injury from a penile hair ring, or urethral necrosis associated with the presence of a urethral calculus (Figure 15.17). The bull's ability to deposit semen properly in the cranial vagina may be compromised depending on the location of the fistula [33].

Paraphimosis

Paraphimosis, the inability to retract the penis into the preputial cavity, may occur following penile laceration or preputial trauma. Edema effectively reduces the diameter of the preputial orifice and the non-erect penis remains exposed beyond the preputial orifice. The exposed preputial and penile epithelium desiccates rapidly and the superficial layers become necrotic and slough. The exposed penis is typically discolored and assumes a mild corkscrew orientation (Figure 15.18). Paraphimosis resulting from breeding injuries warrants a grave prognosis and treatment must be initiated early to be successful. Apply emollient ointments and humectants to protect the exposed skin and cover the damaged tissues with a length of orthopedic stockinette or other light bandage material. Frequent bandage changes and fresh application of dressings combined with daily cold water hosing should continue until the penis can be retracted into the prepuce. Continue preputial lavage with antiseptics and application of antibiotic ointments or emollients for at least a week after the penis is returned to the sheath. Even with aggressive early treatment, return to service is unlikely and the chance for a successful outcome decreases the longer treatment is delayed following injury.

Paraphimosis may sometimes be associated with the presence of a penile papilloma on the distal penis large enough to prohibit retraction through the preputial orifice. Surgical removal of the wart can be curative.

Penile Hematoma (Rupture of the Tunica Albuginea of the Penis)

While any extravascular accumulation of blood in the vicinity of the penis could technically be termed a penile hematoma, in the bull the terms "hematoma of the penis" and "penile hematoma" are usually reserved to describe the breeding injury that results in rupture of the tunica albuginea of the CCP. The penis of ruminants is well equipped to withstand high intrapenile pressure created within the CCP. At the time of erection, venous outflow to the CCP is obstructed and contraction of the ischiocavern-



Figure 15.17 Urethral fistula. *Source:* Courtesy of Richard Hopper.



Figure 15.18 Paraphimosis following traumatic injury to the preputial trauma. The exposed penile and preputial epithelium desiccates rapidly and the free portion of the penis forms a spiral.

osus muscles increases pressure within the penis to 14 000 mmHg (1.87 MPa) or greater [3]. Pressures of this magnitude are easily contained by the thick fibrous tunica albuginea of the penis which encompasses the CCP. However, if a cow or heifer collapses during coitus or if an ill-timed breeding lunge accidently forces the erect penis against the escutcheon, sudden angulation of the penile shaft may increase intrapenile pressure to greater than the 70 000 mmHg (9.3 MPa) required to rupture the tunica albuginea [34]. Although occasionally seen at other sites [35, 36], rupture of the tunica albuginea almost inevitably occurs on the dorsum of the penis opposite the attachment of the retractor penis muscles on the distal bend of the sigmoid flexure.

Rupture of the tunica albuginea is accompanied by extravasation of the blood contained in the CCP and formation of a hematoma which is visible as a symmetrical enlargement at the site of the rupture. While the erect penis contains a relatively small quantity of blood (about 150–250 ml), the hematoma may grow from comparatively small to quite large if repeated sexual stimulation results in additional attempts at erection and continued leakage of blood through the rent. In some cases several liters of blood may accumulate in the peripenile tissues and form a large symmetrical swelling dorsal to the penis near the neck of the scrotum (Figure 15.19). Hematoma of the penis is diagnosed based on physical examination findings, occasionally supplemented by ultrasound, and may be differentiated from retropreputial abscess by the typical location and symmetry of the swelling.

Following rupture of the tunica albuginea, ventral migration of blood and edema within the peripenile elastic tissues often results in a secondary prolapse of preputial tissues through the preputial orifice. These mild to moderate preputial prolapses may have a distinct bluish tinge as a result of subcutaneous blood and may be the first sign noticed by the owner or manager of the bull (Figure 15.20).

Rupture of the tunica albuginea is seldom life-threatening, but the injury and the complications that follow can result in permanent loss of reproductive function. Potential complications following penile hematoma include abscess formation at the site of the hematoma, formation of adhesions between the penis and peripenile tissues, development of vascular shunts between the CCP and the surrounding vasculature, injury to the prolapsed preputial tissues, and destruction of the dorsal nerves of the penis. Injury to the dorsal nerves of the penis at the time of injury or by entrapment injury as scar tissues remodel can result in loss of sensation to the distal penis, rendering the bull unable to breed by natural service. Even following apparently successful management and resolution, recurrence of injury may occur during subsequent attempts at breeding [17].

Case management options following rupture of the tunica albuginea include salvage for slaughter, surgical removal of the blood clot coupled with repair of the rent, or medical management. If the injury is recognized early, surgical removal of the hematoma and closure of the rent increases the likelihood for restoration of breeding ability and decreases the incidence of other post-injury sequelae. Surgical repair of hematoma of the penis and postoperative care is covered in Chapter 19. Medical management is advocated when diagnosis has been delayed or when the economic value of the bull will not justify the expense of surgery and aftercare. Non-surgical management includes



Figure 15.19 Hematoma of the penis (rupture of tunica albuginea). Note the location of the swelling, dorsal to the penis and cranial to the scrotal neck. *Source:* Courtesy of Richard Hopper.



Figure 15.20 Preputial prolapse associated with penile hematoma due to rupture of tunica albuginea of the penis.

broad-spectrum antibiotic coverage to decrease the likelihood of abscess formation, twice-daily cold water hydrotherapy of the affected area, local treatment of the secondary preputial prolapse, and strict enforcement of sexual rest for 60–90 days.

Erection Failure

Inability to achieve or maintain penile erection (*impotentia erigendi*) precludes natural service. A history of failure to impregnate females in the breeding pasture or observation of unsuccessful breeding is often the presenting complaint. A well-taken history including previous breeding performance, breeding injuries, and the owner's description of the appearance of the bull at the time of attempted coitus are valuable, but observation of the penis during an attempt at erection is a required element for diagnostic evaluation. Use of the electroejaculator to induce erection may be useful but a controlled test mating is preferred.

Because painful stimuli from the spine, rear limbs, or pelvis may interfere with the willingness or ability of the bull to achieve erection and complete the breeding act, a physical examination of the bull at rest and in motion is mandatory. Appropriate management or correction of painful musculoskeletal and spinal conditions may be useful and return some bulls to breeding soundness.

True erection failure may involve disruption of vascular components of the erection mechanism or failure of the corpus cavernosum to fill completely.

Erection Failure Due to Vascular Shunts

Sexual stimulation of the bull is followed by increased blood flow through the crura of the penis and into the CCP. This mechanism may be mimicked by stimulation with an electroejaculator. As discussed previously, the tunica albuginea encases the erectile tissues in the CCP and there are normally no functional venous outlets along the body or shaft of the penis at the time of erection, allowing the intact tunica albuginea to effectively maintain a closed hydraulic system to contain the extraordinary pressures generated as the ischiocavernosus muscles rhythmically contract against the blood-filled crura of the penis [2, 4]. If the integrity of the fibrous tunica albuginea is compromised, anastomoses between the CCP and the surrounding peripenile vasculature may form and provide an escape route for blood contained in the CCP. Should this occur, pressure sufficient to achieve or maintain erection cannot develop [28, 37]. Communication of the CCP with the corpus spongiosum penis will produce a similar result because venous drainage of the CSP is not occluded during erection [29, 38]. Formation of vascular shunts may follow traumatic disruption of the tunica albuginea or be associated with a congenital weakness of the tissues of the tunica albuginea.

The presence of vascular shunts as a cause of erection failure can be suspected based on findings of an observed test mating, and confirmation depends on demonstration of the vascular communication of the CCP and surrounding vasculature or CSP using radiographic contrast studies. Cavernosography (Figure 15.12) utilizes water-soluble radiographic contrast media (Renografin 76, Squibb Diagnostics, New Brunswick, NJ). Best results are obtained with the bull restrained in lateral recumbency on a tilt table. Extend the penis and place towel clamps under the dorsal apical ligament and apply sufficient traction on the towel clamps to maintain extension of the penis. A length of suture or umbilical tape should be attached to the towel clamps to keep the hands of the assistant out of the radiograph beam. At the same time, place a 30-cm loop of suture through the skin under the retractor penis muscles and apply traction to pull the more proximal portion of the penis away from the abdomen for better radiographic visualization. Make an initial scout film without contrast media to establish appropriate radiographic settings. While utilizing the towel clamps to extend the penis and the loop of suture under the retractor penis muscles to pull the penis from the abdomen, insert a 16-gauge needle into the CCP on the dorsum of the free portion of the penis and attach an infusion set of sufficient length to keep the operator's hands out of the radiographic field (Figure 15.21). Inject saline into the CCP to ensure proper placement, and once proper needle placement is ascertained, inject 15 ml of the radiographic contrast media and begin the radiographic series. Have the assistant slowly inject additional contrast media over the next 60 seconds as serial radiographs are taken of as much of the penis as possible. Take enough exposures to allow visualization of the free portion of the penis and penile shaft to the level of the distal sigmoid flexure [39].

Formation of vascular shunts is recognized as a potential complication following rupture of the tunica albuginea as described under the section on penile hematoma. Anastomoses which develop following disruption of the integrity of the tunica albuginea and subsequent penile hematoma formation are located at the site of injury, the dorsum of the penis at the level of the distal bend of the sigmoid flexure (Figure 15.22). Penetrating injury to the tunica albuginea at sites other than the location of a penile hematoma may also be followed by shunt formation. Surgical correction of vascular shunts that occur following either type of trauma may be successful.

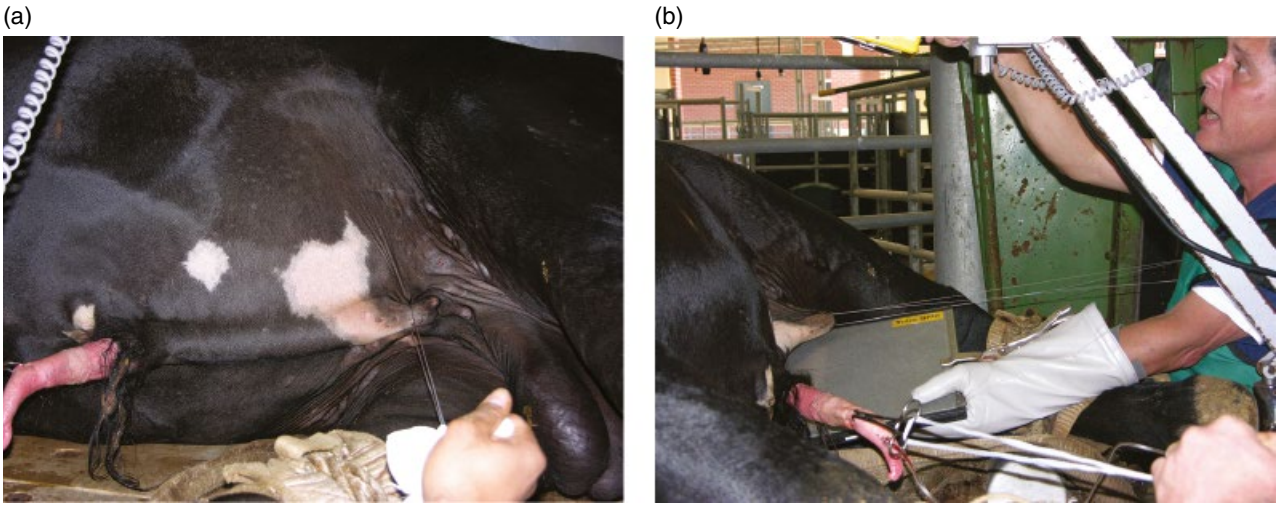


Figure 15.21 Bull prepared for cavernosography. Note suture placed through the abdominal skin and under retractor penis muscles to pull the penis away from the abdomen (a). As the bull is positioned, an extension set is attached to a hypodermic needle placed into the CCP of the free portion of the penis, the penis is held in extension with a towel clamp placed under the dorsal apical ligament, and traction is applied to the preplaced sutures under the retractor penis muscles (b).

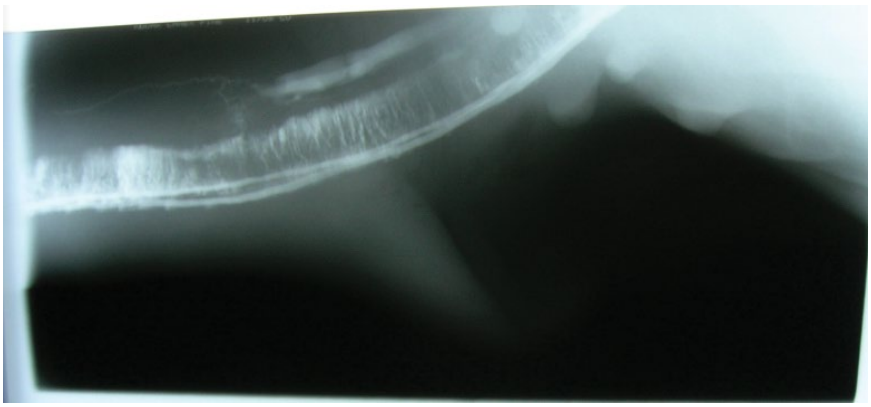


Figure 15.22 Cavernosogram of the penis of a bull demonstrating escape of contrast media into the peripenile circulation at the level of the distal sigmoid flexure. *Source:* Courtesy of Dwight Wolfe.

Cases of erection failure due to multiple vascular shunts involving defects in the tunica albuginea of the free portion of the penis are thought to be the result of a congenital weakness in the structural integrity of the tunica. In such cases there is no history of penile trauma or injury. Affected bulls fail to achieve erection following sexual stimulation and may have bluish discoloration of the penile or preputial skin when attempting to breed or when attempts are made to induce erection with an electroejaculator. Discoloration is the result of blood exiting the CCP through the peripenile vasculature. Multiple distal vascular shunts are most often diagnosed in bulls during the first breeding season. Vascular shunts in the distal penis are readily demonstrable with cavernosography (see Figure 15.13). Unlike vascular shunts that form secondary to traumatic disruption of the tunica albuginea, surgical correction of multiple congenital shunts is unsuccessful.

Filling Defects of the CCP

Erection and maintenance of the normal penile form is dependent on complete distension of the unobstructed cavernous spaces with blood under pressure. If the cavernous spaces are blocked sufficiently to prevent blood from completely filling the CCP, engorgement of the penis can only progress from the proximal portion of the penis to the area of the filling defect [40]. The clinical presentation depends on the location of the obstruction. Complete obstruction of portions of the CCP may result in extension of the penis without erection of the portions distal to the blockage (Figure 15.23). Partial blockade of the cavernous spaces in the distal free portion of the penis may sometimes cause the erect penis to deviate ventrally or laterally.

Cavernosography can demonstrate filling defects in portions of the penis located distal to the sigmoid flexure.



Figure 15.23 Failure of the distal portion of the penis to become erect due to occlusion of the cavernous spaces of the corpus cavernosum penis.

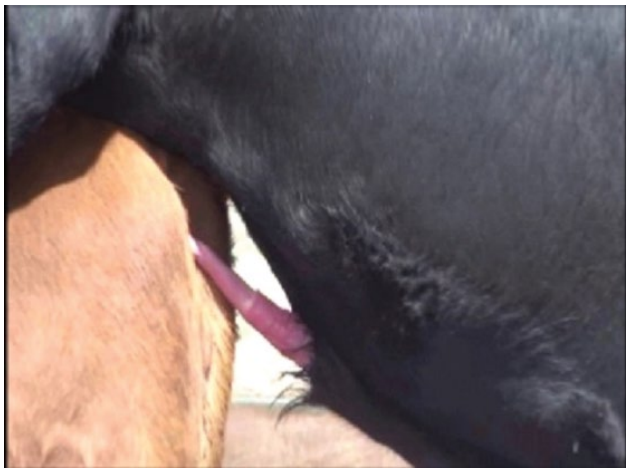


Figure 15.24 Failed attempt at copulation due to denervation injury of the penis. During repeated breeding attempts during test mating, the bull was unsuccessful in locating the vulva with the penis. Note the location of the penis beside the tail head of the cow.

Filling defects may be congenital, secondary to fibrosis following trauma, or subsequent to cavernositis.

Denervation Injury

When mounting an estrus female the bull must position himself to make searching motions and use the penis to locate the vulva in order to make intromission. Sensory innervation to the glans and free portion of the penis is necessary for the bull to align the penis, achieve intromission, and successfully complete the breeding act. Without sufficient sensory innervation of the distal penis the bull is unable to locate the vagina and the coital act cannot be completed [41].

Sensory input from the distal penis is transmitted by branches of the paired dorsal nerves of the penis through the pudendal nerves to reach the spinal cord and brain. Although disruption of any portion of the neurologic pathway could result in loss of sensation, damage to the dorsal nerves of the penis is the most likely etiology of penile desensitization. Denervation injury does not interfere with erection, but affected bulls are unable to breed by natural service and usually cannot be successfully collected with an AV. Semen collection with an electroejaculator for artificial insemination is possible.

An observed test mating utilizing an estrus female in a confined area with adequate footing remains the best method to evaluate function of the dorsal nerves of the penis. Successful intromission and ejaculation definitively demonstrate normal nerve function [17]. In bulls with impairment of sensation to the distal penis, erection is not impaired but the searching motions made with the penis are ineffective and the bull will be unable to achieve intromission and complete the breeding act. Affected bulls often place the penis to the side of the tail head, on the cow's hip, or below the vulva near the udder (Figure 15.24). Observation of typical signs at the time of test mating coupled with a history of reproductive failure is usually sufficient for diagnosis, but definitive electrodiagnostic testing of nerve function [39, 42] is available at some veterinary teaching hospitals (Figure 15.25).



Figure 15.25 Electrodes and stimulator in place for nerve conduction study of dorsal penile nerves. Electrical stimulation applied through the electrode on the free portion of the penis is recorded as the signal passes the proximally placed electrodes.

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16

Management of Lameness in Breeding Bulls

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Introduction

The following discussion of lameness issues in cattle is not intended to be a recipe for how to resolve a particular problem associated with lameness. It is a compilation of knowledge gained from 40 years of serving the beef cattle industry. Lameness evaluation is an analytical process, to be taken one step at a time, giving consideration to history, age, breed, sex, environment, intended use, and nutritional state. Conducting a proper examination on the bovine animal is no different from what is done in the equine species, though the examination of cattle takes more time and requires better restraint. Bovine lameness examination requires a pen or alley with a hard surface and a tilt table or tilt squeeze chute. Proper restraint reduces the chance of injury for both the patient and the examiner. In practices that do not have proper facilities or methods of restraint, often the diagnosis of lameness comes down to a good guess (Figure 16.1).

Most of the literature available is derived from treatment of dairy cow lameness; very little has been reported dealing with beef cattle. By the end of the chapter, the reader should have a better appreciation of lameness conditions in beef cattle and their expected response to treatment.

Bovine lameness examinations must begin with a good history that considers duration of lameness, observation of irregularities, and the production parameters involved. During visual examination, the whole body and every limb must be evaluated before focusing on the affected limb. For instance, while examining an animal for a suspected subsolar abscess in a back hoof, do not miss the laceration on the pastern of the supporting limb and subsequent swelling indicating a septic flexor tendon sheath. Watching the animal in motion may help in ruling out proximal versus distal limb problems. Observing the entire animal is important as sometimes other limbs may be affected by the primary lameness or may contribute further to it (Figure 16.2).

Getting Back to Basics in a Lameness Examination

Unfortunately, economics dictate what practitioners are able to offer the client and the patient. Additionally, some clinical options available to other species are just not practical in bovine practice. If the problem is not obvious after visual examination, the next step is to properly restrain the patient on a hydraulic tilt table or tilt chute. Casting the animal is another option, but it is difficult without an abundance of labor and may not allow for an accurate examination. It is possible to cause harm or even create a new injury by tying a leg in a squeeze chute with the good intention to examine a lesion more closely. Since approximately 70% of bovine lameness is in the hoof, hoof testing while the patient is in lateral recumbency is an important part of the examination. If hoof testing reveals a painful area, explore the area by paring the hoof and examine any cracks, crevices, or wall separations. Often aggressive trimming of the sole will provide evidence of a puncture, laceration, or developing crack that can lead to an abscess. If no pain is elicited on hoof testing and no direct visual abnormalities are noted, attention should be directed further up the limb. Each joint should be palpated and individually flexed and extended, including the shoulder or hip. Most often, lameness caused by a joint in the lower limb can be identified by flexion or extension of the joint, eliciting a painful withdrawal of the limb by the animal. The flexor and extensor tendons must be evaluated for evidence of sepsis or rupture. If the examination at this

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine



Figure 16.1 Looks can be deceiving – a calf with spastic paresis.



Figure 16.2 Cellulitis involving the right forelimb and interdigital fibroma on the left forelimb.

point has still not revealed the cause for the lameness, the animal may have to undergo local anesthetic nerve or tissue infiltration in order to isolate the painful area.

Facilities for Performing the Lameness Examination

Diagnosis of lameness in the field is not ideal, and the practitioner must consider possible risks to themselves, the client or caretaker, and the animal. Having a proper facility to diagnose and provide treatment to lame individuals makes the opportunity much more appealing. Let us consider what would be the ideal setting for evaluation of bovine lameness.

The most desirable area to evaluate a bovine animal in motion is on a hard-surfaced alley, free of rocks or other irregular debris, with access on either end to stimulate the animal to travel back and forth (Figure 16.3). Additionally, an alley or chute leading to a tilt table or a tilt chute allows the practitioner to get a closer look at all limbs, hooves, and back while preparing to place the animal in lateral recumbency (Figure 16.4).

A hydraulic tilt table is preferred as it allows full access to the upper part of the limbs and allows the animal to be positioned in either left or right recumbency (Figures 16.5 and 16.6). Most hydraulic tilt chutes only allow the animal to be placed in a single position. Animal restraint on a tilt table is also easier due to the application of belly bands for support and leg ropes used to restrain limbs on the table. Leg ropes should also be used with a tilt chute, especially the down forelimb (Figure 16.7).

Placement of the animal on a tilt table is an orchestrated team effort, usually requiring at least two people. There is inherent risk in releasing the restraints at the end of the examination or procedure. Any bovid that is expected to be in lateral recumbency for longer than 15 minutes should be fasted for at least 24 hours before the procedure to reduce possible development of rumen tympany. Also, during lateral recumbency, the animal should be continually observed for any evidence of regurgitation, especially if sedation is used. If regurgitation occurs, the animal should be immediately returned to an upright position (Figure 16.8).

Local/Regional Anesthesia

The use of local anesthetics is helpful in isolating pain in the lameness examination, but proper restraint in lateral



Figure 16.3 Nice alleyway for evaluation of lameness, with room to turn the individual and observe his movement.



Figure 16.4 Crowd alley leading to hydraulic table that enables practitioner to fully evaluate lameness up close.

recumbency is required for administration. The application of anesthetics should only be done after local examination of the affected area and any other diagnostics (including hoof testing, radiography, and ultrasound) have been performed. Lidocaine is used because of its rapid onset of action (3–5 minutes) and short duration (20–30 minutes) (Figure 16.9).

Most often the first anesthetic administered will be a vascular perfusion of all structures distal to and including the



Figure 16.5 Bull prepared for placement in lateral recumbency on hydraulic tilt table.



Figure 16.6 Bull in lateral recumbency on hydraulic tilt table. Observe the leg restraints applied to this case.



Figure 16.7 The down forelimb tied in a tilting hydraulic chute to discourage repositioning of the animal while restrained in the chute.



Figure 16.8 Evidence of rumen tympany and/or regurgitation of rumen contents should lead to immediate removal of animal from lateral recumbency.



Figure 16.9 Vascular perfusion used for local anesthesia as well as treatment.

fetlock. For regional limb perfusion, place a tourniquet (using IV tubing or bicycle inner tube) proximal to the fetlock. In a limb free of edema, wrap tightly enough to identify the dorsal digital vein and administer 15–25 ml of lidocaine. Maintain the tourniquet for 10 minutes before releasing the animal to observe for lameness. If no improvement in gait occurs, the animal is restrained again. Regional whole limb perfusion or a four-point block may be used to isolate lameness below the elbow or stifle. To perfuse the whole limb, 30 ml of lidocaine in 50 ml of saline is administered with a tourniquet above the carpus or tarsus. This can be performed standing in a squeeze chute. The practitioner may also consider the use of intra-articular anesthesia for individual joints; 10 ml of lidocaine is sufficient for each joint except the stifle, which usually requires a larger volume.

In beef cattle with forelimb lameness, if the cause does not appear to be in the hoof, it is often in the shoulder. This is suspected to be due to inadvertent abduction during mounting or due to falls and displacement of the limb laterally. Rear limbs can be difficult to evaluate and require more patience and effort to examine. The coxofemoral joint must not be overlooked as a cause of lameness in the rear limb. These too can be injected with anesthetics to rule in or out lameness.

Back Injuries

The back is often forgotten as a cause or contributor to lameness, particularly in the bull. Close evaluation may lead to subtle findings such as a shuffling of the rear limbs or muscle atrophy of the lumbar muscles. Pelvic injuries can happen in both sexes of cattle, and sacroiliac injuries supporting ligamentous injuries and fractures to the ilium and ischium are possible [1]. Diagnostic anesthesia in the lower back is rarely effective due to the involvement of multiple structures in most back injuries. Acupuncture and chiropractic examinations may be of value in isolating the problem. Since most radiographic equipment available in practice cannot penetrate the body mass of most mature cattle, radiographs of the spine are of little value. Sometimes, all we can do is make an educated guess and come up with a conservative therapeutic plan.

Advanced Diagnostics

Many practices now have digital radiography and digitally enhanced imaging to allow the practitioner the opportunity to clearly identify problems that could only be guessed at a few years ago. It can be a bit challenging to position the animal to get a good image of some areas, but with experience and sometimes sedation of the patient, a good study can be performed.

Ultrasound is used to evaluate soft tissue injury of ligaments, menisci, and tendon sheaths. Proper technique and an experienced skill set are necessary to fully appreciate

abnormalities associated with lameness. An inexperienced operator or one with less than optimal knowledge of bovine anatomy may lead to misdiagnosis of some conditions.

Arthrocentesis is another valuable diagnostic tool for evaluation of lameness directly associated with a joint. Color, viscosity, protein content, and cellularity are used to diagnose the health of the joint and can offer a reasonable prognosis. This procedure can be performed in most practices with laboratory access. Evaluation of cell types and protein level found within the synovial fluid is most important. Cellularity should be less than 1000 cells/μl, and protein should be less than 2.5 g/dl in the normal joint.

Laminitis

Seventy percent of the lameness cases observed in beef cattle practice are associated with the hoof. Fifty percent of these cases are diagnosed with chronic laminitis, the vast majority of which are the chronic subclinical form. Acute laminitis is caused by an acute metabolic or systemic illness and is characterized by reluctance to stand, arched back, and the appearance of walking as if they were on a very hot surface (Figure 16.10).

There are no outward lesions expressed or seen on the soles of the hoof, although inflammation may be observed in the coronary band and a digital pulse may be present. Subacute laminitis doesn't usually express itself until several weeks after the insult, and the symptoms observed are sole hemorrhage and discoloration of the white line and sole tissue.

Subclinical laminitis is due to periodic upsets in normal body function. A few scenarios that can predispose to chronic laminitis include:

- Mismanagement of the young growing calf
- Bulls on gain test
- Cattle being prepped for sale
- Cattle breeds involved with progressive genetic improvement
- Cattle being fitted for show
- Dairy cows being fed for maximum milk production

The general appearance of a hoof with subclinical laminitis is one that flares out at the wall with a flattened sole. Closer examination reveals white line separation with abscessation, vertical and horizontal wall fissures, heel erosion, and subsolar ulcers [2]. Horizontal lines (known as hardship lines) may be observed in a portion or the entire hoof wall. Over time, more than one hoof can be affected. Since hoof overgrowth is the number one initiator of lameness, cattle with chronic laminitis should have regular hoof trimming to maintain proper hoof health [3]. The vascular damage that occurs during episodes of subclinical laminitis can lead to a cascade of hoof problems, discussed in the following sections [4] (Figures 16.11 and 16.12).

Treatment of Solar Injuries

Many predisposing factors have been mentioned that lead to laminitis and play a part in subsolar injuries. The metabolic effects of subclinical acidosis and laminitis can affect normal hoof growth and lead to white line separation, toe ulcers, heel erosion or ulceration, and sole ulceration. Puncture wounds and traumatic concussive injury are other contributing factors to subsolar abscess formation. Without proper treatment, these conditions may lead to damage of deeper structures including pedal osteitis, infection of the navicular bursa, sepsis of the distal interphalangeal (DIP) joint, and septic tenosynovitis of the digital flexor tendon sheath.



Figure 16.10 Acute laminitis in a Hereford bull on self-feeder.



Figure 16.11 Severe white line disease and hoof crack as a result of chronic subclinical laminitis.



Figure 16.12 Subclinical laminitis with hardship lines present as horizontal grooves in hoof wall.



Figure 16.13 Vertical fissure in hoof wall due to subclinical laminitis.

Treatment of complicated subsolar abscesses and white line disease involves complete and total curettage of the overlying sole or hoof wall and removing all necrotic debris from the affected area. Exploration of all tracts is necessary to evaluate if deeper structures are involved. If sepsis of the pedal bone or coffin joint is suspected, radiographs should be performed at this time. Parenteral antibiotics and bandaging with astringent antiseptic compounds should be incorporated into a treatment plan when possible. While some practitioners elect to leave sole abscess lesions exposed, it is my opinion that these wounds heal better in beef cattle when protected. The application of a hoof block to the supporting claw prevents the affected claw from bearing weight and allows quicker healing due to the lack of contact with sole surface.

Vertical Hoof Cracks

The prevalence of hoof cracks appears to be increasing, most likely related to genetics and intensive management. Subclinical laminitis has been suspected as an underlying cause in many of these cases. These cracks can affect one or multiple claws (Figure 16.13).

Lameness is caused by instability in the hoof wall as weight is shifted to the wall. Debris and soil may be packed into the crack, inducing abscessation with encroachment on the sensitive lamina. Remove all debris through careful curettage. If any debris is left before immobilization, it can lead to sepsis of the laminae. If debridement of the crack results in penetration of the laminae, antibiotic bandages should be applied until the wound completely heals and the crack is dry and hard.

To stabilize a hoof crack, drill horizontally across the crack and place stainless-steel wire across the defect in a bootlace pattern. Application of an acrylic compound into the crack and around the wire will bond the hoof. Alternatively, the crack can be filled with fiberglass cloth and acrylic compound that bonds to the hoof wall. This will bridge the crack and stabilize it, allowing new normal hoof wall to be produced at the coronary band. Newer products are now available that come with an applicator that allows direct application to the affected area without the need for any other material to support the repair.

If there is radiographic evidence of abscessation or osteomyelitis, open and debride the area to allow for healing. Curettage of the coffin bone and removal of bony fragments may have to be performed. To alleviate pain during

movement, apply a hoof block to the healthy claw or consider using a splint. Systemic antibiotics are always indicated in situations involving deep sepsis of the hoof, and localized vascular perfusion can aid in increasing antibiotic concentrations within the area. Potassium penicillin, ampicillin, and tulathromycin are commonly used for vascular perfusion. Common side effects of tulathromycin include vasculitis and localized tissue edema. Medicated bandages, analgesics, and support bandaging of the contralateral limb should also be considered.

Septic Arthritis of the Distal Interphalangeal Joint

Septic arthritis of the DIP joint is a common sequela to chronic infectious processes involving the hoof and/or interdigital space. Examination findings include a swollen coronary band with a draining tract occurring at the dorsal aspect of the extensor process, diffuse swelling of the pastern area, and partial or complete non-weight-bearing lameness. Radiographs will reveal osteomyelitis of the coffin joint and increased joint space, with osteolysis of the second and third phalanx (Figure 16.14).

To block the hoof and provide postsurgical analgesia, perform regional limb perfusion using a long-acting local anesthetic, such as mepivacaine or bupivacaine. The following surgical approach to the DIP joint is preferred over the abaxial approach because it allows better exposure of the joint [5]. Closely trim the sole and heel area and disinfect with surgical scrub and alcohol. A full-thickness incision is made through the heel and continuing through the deep flexor tendon to expose the intra-articular area between the navicular bone and the proximal extent of the caudal portion of the coffin bone. It is recommended that the navicular bone be removed. A half-inch drill is used to facilitate curettage of the joint surface, drilling from the heel incision toward the extensor process and following the surface of the joint [6]. Further debridement of the joint is necessary using a large bone curette. Curettage is complete when you can't remove any more necrotic bone. Use a squeeze bulb to lavage the surgical site with a broad-spectrum antibiotic or antiseptic saline solution. Place a wooden block on the healthy claw, pack the joint with medical-grade honey, and bandage the hoof [7] (Figure 16.15).

Parenteral antibiotics are administered daily, and bandage changes and joint lavage are performed every other day up to four times. Beads of bone cement mixed with an antibiotic can be placed in the joint space to facilitate healing, but they should not be placed until there is a healthy granulation bed and no more purulent discharge. When the surgical wound has closed with granulation tissue, the limb is casted above the fetlock for five to six weeks. After the cast and hoof block are removed, the animal should have a recuperative period. The patient should show at least 80% improvement in lameness over the initial presentation after the convalescent period; some lameness is to be expected.



Figure 16.14 Sepsis of the DIP joint with sequestrum formation.



Figure 16.15 Dressing change post DIP joint currretage. Notice the gauze packing ingress at location of the extensor process of the third phalanx and eggress at the heel bulb.

Corkscrew Claw

Corkscrew claw (CSC) is observed in the young adult beef animal and usually involves the lateral claw of both hindlimbs. In some breeds, such as Brahman and Brahman crosses, it can occur on the medial claw of both forelimbs. Because CSC can be seen in calves and yearlings, some practitioners believe that this is a heritable condition. However, it appears that this is a management-induced problem, in our opinion, even in younger animals. Offering overabundant feed can lead to rapid skeletal growth and subsequent epiphysitis and rotational limb deformity. This results in abnormal load bearing and compensatory changes in the hoof. Therefore rapidly growing animals may express CSC. To prevent structural changes to the phalanges secondary to CSC, corrective hoof trimming must occur during the active growth phase. In the fully grown adult, CSC cannot be cured, only managed [8] (Figure 16.16).

It is unknown whether bone remodeling in the distal phalanx is the cause or effect of CSC. In some patients with CSC, the second and third phalanges become misaligned and the third phalanx becomes narrower with an abaxial curvature. This leads to abnormal load bearing, which affects the white line and leads to separation. In other patients with CSC, the growth rate of the middle to caudal portion of the wall is faster than the fore portion of the toe, which causes the animal to bear weight on the abaxial wall, predisposing to bruising, abscessation, and sole ulcers.



Figure 16.16 Corkscrew claw on the lateral claw of a Brahman bull.

Correction of CSC involves balancing the weight-bearing surface of both claws. It is best to trim excess from the medial claw just until the white line is apparent. Next shorten the affected claw to the same length as the normal claw. Remove the upward deviation and rotation of the wall with a grinder or nippers. The heel is often higher in the CSC and it should not be lowered. Bevel the sole to encourage wall contact with the sole surface. At this point, there may not be very much area for bearing weight, but it will encourage more favorable wear for the hoof wall. Corrective hoof trimming should occur every four to six months.

Interdigital Dermatitis, Digital Dermatitis, and Interdigital Pododermatitis

The hoof problems of interdigital dermatitis, digital dermatitis, and interdigital pododermatitis are often lumped together, but each condition has its own etiology, tissue involvement, and disease progression. Interdigital and digital dermatitis are often not serious problems unless left to become chronic. Interdigital pododermatitis can progress rapidly and cause serious economic loss if left untreated.

Interdigital dermatitis is a localized inflammatory process involving the interdigital space. In the early stages, the skin has a greasy appearance and some erosion can occur, particularly in the heel bulb area. Digital dermatitis (hairy heel wart) is another low-grade inflammatory process with pronounced change when allowed to become chronic. The epidermal layer becomes overproductive, at first producing excessive hair growth that stands out from the skin, and progressing to small papillary projections giving the appearance of wart-like growths. As the condition becomes chronic, the dermis can become very proliferative [9], making for an unsightly lesion. These conditions respond well to treatment via footbath. Care should be taken in the choices of medications recommended in footbath treatments as some products may cause environmental contamination with improper disposal [10]. A copper sulfate solution is considered environmentally safe. In chronic lesions that are extremely proliferative, surgical resection and topical treatment may have to be provided before resolution can occur.

Interdigital pododermatitis, commonly called foot rot, can be an acute, aggressive infection that progresses to deeper structures quickly. While systemic antibiotics are rarely indicated in the treatment of digital or interdigital

dermatitis, they should always be administered in patients with foot rot. Bandaging with a broad-spectrum antiseptic or antibiotic is recommended when treating each of these three conditions.

Interdigital Fibroma

Interdigital fibroma is a condition most often observed in heavier weight beef breeds, particularly older bulls with widely placed claws. Occasionally, cows will develop fibromas as well. If the condition is allowed to persist, the "corn" can become ulcerated and infected due to continued contact with the ground (Figures 16.17, 16.18 and 16.19).

Surgical removal is necessary to resolve the fibroma. Anesthesia of the interdigital area can be provided by four-point block or the use of a tourniquet and intravascular anesthesia. Removal of the prolapsed tissue is accomplished by sharp dissection followed by extraction of the fat pad. Cautery of the vascular bed is sometimes required to control excessive hemorrhage. A bandage is applied with a sterile gauze pack and changed after three days. As an aid to healing, especially in the individual with splayed toes, it is helpful to wire the toes together to reduce swelling and decrease granulation bed formation. The second bandage can be removed in 4 days, with complete healing expected within 10 days of surgery.



Figure 16.17 Digital dermatitis (hairy heel wart).



Figure 16.18 Interdigital fibroma can be misleading; this one does not appear to be significant until observed from the solar surface.

Distal Limb

Lameness involving the distal limb usually has a traumatic origin. Most often, acute and severe lameness can be attributed to a severe concussive force, entanglement or entrapment of the lower limb within a fence or handling device, or foreign body penetration of the skin with invasion of deeper structures. Young cattle are predisposed to joint sepsis due to hematogenous spread of bacteria from the umbilicus, digestive tract, or lungs in times of general immune compromise [11].

Joint sepsis often presents as severe lameness of several days' duration. Many cases will not respond to broad-spectrum antibiotics administered by the owner. The animal will have an elevated heart rate and usually an elevated rectal temperature. Thorough examination should be performed under appropriate restraint, preferably on a hydraulic tilt table. Radiographs should be performed to detect any concurrent fractures. Both systemic and local antibiotics via vascular perfusion should be administered. In severe cases, joint lavage must be considered. In these cases, it may be difficult to lavage the joint space due to fibrin accumulation within the joint. Fibrin deposition seems to increase with subsequent flushes, and in those cases that are slow to respond to treatment, arthrotomy or arthroscopic surgery may be the only option to remove the fibrin deposits from the joint. Surgical drain placement is not advised due to the risk of contamination even with proper bandaging. In cases where there are financial constraints and osteomyelitis is already present, aggressive curettage of the area may be the only alternative. Active lavage can be incorporated with sterile bandaging to provide continued protection, although compression bandaging may suppress desired drainage [12]. When there is marked reduction in exudate coming from surgical wounds, polymethyl methacrylate (PMMA) beads impregnated with an appropriate antibiotic might be considered for placement in the remaining joint space. When the beads are used, the goal is joint ankylosis, which cattle seem to take well. When wounds no longer need attention,

it is best to immobilize the limb in a cast, with consideration to using transfixation pins. Cattle tolerate the process well as long as a support bandage protects the contralateral limb. Due to the expense of these procedures, clients may only elect to have these procedures done for cattle with high economic or emotional value.

Sequestra should be surgically removed. Most often, these occur on the lateral aspect of the metatarsus and are directly related to trauma from kicking a hard object like a gate or post or entrapment of the limb within a confined space (Figure 16.20). On presentation, most cattle with a sequestrum will have an associated draining tract. Surgical removal can be performed under vascular anesthesia and sedation while the animal is restrained in lateral recumbency unless there is extensive bony proliferation that should also be removed, in which case general anesthesia should be considered. A surgical drain should always be incorporated in the closure, as there is virtually no way to close the entire dead space after removal of the sequestrum. The drain should be removed four to five days after surgery and recovery may take up to two weeks.

Sepsis of the superficial and/or deep digital flexor tendon is usually due to traumatic injury (laceration of the caudal pastern or heel bulb area) or due to extension of deep infection from a chronic hoof rot lesion. Parenteral antibiotics alone will not resolve this problem; surgical drainage is performed and a drain placed from the pastern, through the tendon sheath and above the dewclaw. Extensive and chronic sepsis may require complete resection of the superficial and deep flexor tendons, after which partial closure with packing and drain placement is a must. Active or passive lavage may be provided, with several bandage changes required to resolve the issue. The drain should be removed in five to seven days. Apply a hoof block to the non-affected claw and cast the limb to the carpus or tarsus to alleviate pain and allow better ambulation. After four weeks the cast may be removed. Some deep digital flexor tendons may spontaneously rupture after treatment, which will cause the patient to walk on the heel bulb.



Figure 16.19 Sole view of interdigital fibroma. Notice the ulceration that has occurred due to contact with the ground.



Figure 16.20 Sequestrum formation of the lateral cortex of MTIII in a yearling.

Proximal Limb

Lameness involving the femorotibial joint usually involves a traumatic episode such as a bullfight, breeding injury, or restraint accident. Suspected stifle injuries should be addressed immediately, and treatment should be aimed at decreasing inflammation and reducing the development of degenerative joint disease. Diagnostics often include observation of ambulation and weight bearing, amount of joint effusion present, joint laxity and the direction of laxity, radiographs, and ultrasound (which may not be possible in larger individuals) [13]. Arthrocentesis is used to rule out sepsis, and total cell count should be less than 2500 WBC/μl and protein less than 4.5 g/dl. Ideally, positive pressure joint lavage should be performed with a sterile isotonic fluid solution containing antibiotics and anti-inflammatories [14]. Follow-up therapies may include repeated lavage and regenerative medicine modalities such as plasma-rich protein or stem cell therapy. Strict stall confinement is necessary for healing. Some injuries take up to six months to heal, and severe cranial cruciate or collateral ligament tears may require strict immobilization with a Thomas

splint. These cases should be treated with long-term non-steroidal anti-inflammatory drugs (NSAIDs). Of course, such injuries carry a very guarded prognosis when considering pasture soundness and return to breeding. If only the medial meniscus is involved, arthroscopic surgery may be of benefit in valuable individuals [15].

Lameness associated with the shoulder or elbow joint is often caused by trauma and can have a significant impact on ambulation. Non-weight-bearing lameness usually equates to serious injury such as a fractured scapula or fracture of the olecranon. A lesser degree of lameness may be observed with subchondral cysts or ligamentous injuries to supportive structures. Intra-articular anesthetics may temporarily improve lameness but rarely resolve all of the pain during examination. Treatment of these joints is usually relegated to the use of intra-articular injections unless surgical repair is financially feasible, for example compression plating of a fractured olecranon.

The carpus is probably the least affected large joint in beef cattle. Most lameness associated with this joint occurs in bucking bulls. Degenerative joint disease is observed in older bucking stock due to chronic hyperextension of the carpus when the bull "goes vertical." This condition responds fairly well for a time to intra-articular injections of anti-inflammatories and the administration of NSAIDs.

Upward Fixation of the Patella

Upward fixation of the patella is a fairly easily diagnosed problem in the bovid. Cattle will have a hyperextended hindlimb (extension of tarsus with flexion of fetlock and all distal structure) that appears "locked" and causes the animal to drag the toe while walking. Those that have had the problem for a while will have worn down the toe on the affected limb. Occasionally an animal will exhibit the problem in both limbs, although one limb is often more severe. The condition most often affects females in the last trimester of pregnancy but is also observed in bucking bulls [16] (Figure 16.21).

This condition occurs when the patella is dislocated mediodorsally on the distal femoral trochlea and is treated with a medial patellar desmotomy. It is easier to identify the medial patellar tendon in the standing position. If there is a danger to the surgeon due to temperament, short-acting anesthetics should be used. An appropriate anesthetic would be K-stun (butorphanol, xylazine, ketamine) (Figure 16.22).

The animal is restrained in dorsal recumbency, with the rear limb fully extended and pressure applied to the hoof with forceful downward motion. This will allow for identification of the ligament. For standing procedures, mild



Figure 16.21 Mature bred female experiencing upward fixation of the patella. Notice hyperextension and flexion of the fetlock with dragging of the toe.

K-STUN

Excellent protocol to assist in the restraint of cattle during standing and recumbent procedures

Good induction agent for intubation for use of inhalant anesthesia

Butorphanol	0.025 mg/kg
Xylazine	0.05 mg/kg
Ketamine	0.1 mg/kg

Figure 16.22 Excellent drug combo to facilitate brief examination of procedures in cattle.

sedation with xylazine and a tail hold should be sufficient for restraint. Infuse a local anesthetic, starting approximately 2 cm medial to the tibial tuberosity and include the area of the medial patellar ligament that will be transected. A rib-back scalpel blade or teat bistory is used to transect the ligament. The ligament should be transected in both legs since it is likely that the other limb will be affected. Relief is immediate and little aftercare is required; cattle can be returned to their normal environment.

Rupture of the Gastrocnemius

In commercial cattle, rupture of the gastrocnemius tendon is induced by aggressive mounting behavior by an oversized herd sire or slipping in an already unhealthy individual. It can also be seen in cows undergoing reproductive procedures. If an epidural goes too high, the motor control to the hindlimbs can be affected. As the cow comes out of the chute, she can get her legs caught in a brisket bar or slip on concrete. The injury most often occurs at the junction of muscle and tendon and is quite obvious in the standing

animal. The hock is markedly flexed and may even drop to the ground; the fetlock is simultaneously flexed. The diagnosis can be confirmed with ultrasonography. The condition is usually unilateral, but if the patient continues to struggle the contralateral tendon can also be damaged. Complete rupture of one or both tendons has a grave prognosis. Partial rupture of one or both tendons has a better prognosis but is rarely observed in cattle since most occur in fractious patients and quickly proceeds to complete rupture. Treatment involves a full limb cast or Thomas/Walker splint. Healing usually involves six weeks of immobilization and four weeks of stall confinement. NSAIDs should be used during recovery (Figure 16.23).

Osteochondrosis Dissecans

Osteochondrosis dissecans (OCD) is a common cause of lameness in cattle, particularly those being fitted for show, fed for sale, or being pushed for performance. It is often management induced in cattle with a genetic predisposition, particularly in club calves. Factors leading to OCD development include:



Figure 16.23 Bilateral partial rupture of the gastrocmenius tendon from its associated muscle.

- Excessive feeding with subsequent excessive weight gain
- Lack of proper mineral supplementation leading to mineral imbalance, complicated by increased mineral requirements due to breed variances
- Feeding of distillate products with high sulfur content
- Lack of opportunity for skeletal maturity.

The most common location for OCD in the hindlimb is in the tarsus and the medial malleolus of the tibia; it develops less commonly on the lateral femoral condyle, the tibial plateau, and the medial and lateral trochlear ridges of the talus. In the forelimb, the primary site is the medial surface of the humeral condyle, though osteochondrosis can appear in any joint [17, 18] (Figures 16.24 and 16.25).

Surgical management of OCD and OCD fragments is preferred to slow down or stop the progression of inflammation and subsequent degeneration occurring within the affected joint. When anatomically possible, the OCD lesion should be debrided by curettage and overdrilling or implantation of a compression screw above the lesion (Figure 16.26).

Medical management of cattle with OCD fragments is generally palliative in nature and often does not resolve the lameness, particularly in the growing animal. NSAIDs, stall rest, and intra-articular injection of long-acting anti-inflammatories are utilized. However, as cattle get heavier and more weight is placed on the affected joint, clinical lameness may become worse.

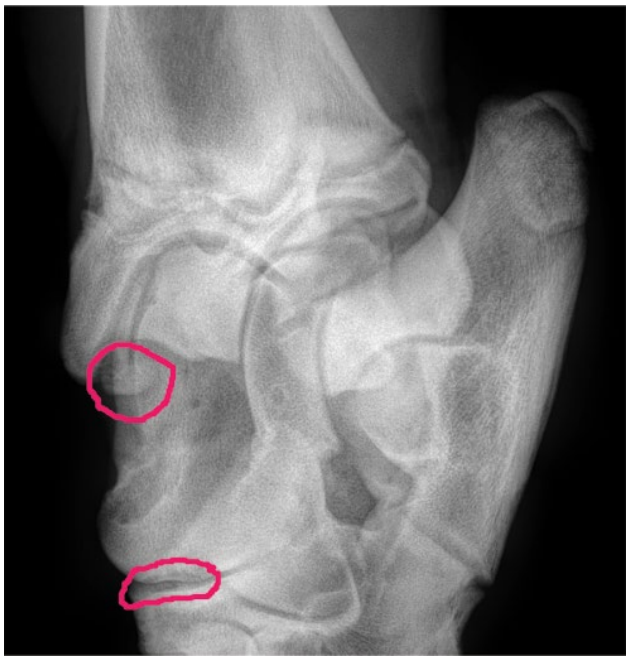


Figure 16.24 Osteochrondrosis lesion in a yearling bull from gain test. Notice the medical malleolar fragment (upper circle) and the cyst in the medial condyle of the talus.



Figure 16.25 Typical appearance of an OCD-affected tarsus (boggy hock).



Figure 16.26 Osteochrondrosis lesion in the talus of a show steer.

Bone Fractures

Fractures in the bovine often occur due to inappropriate management, such as mishandling, crowding, or improper handling facilities. The duty of the practitioner is to evaluate the fracture and determine whether economical repair or salvage is most appropriate. Fractures of the rear limbs often occur due to trauma from other cattle; the tibia is the most commonly affected of the long bones when this occurs.

Physical examination and palpation with manual manipulation of the limb can be helpful to assess the extent of the injury. Radiographs are necessary to determine if the fracture is comminuted or involves a joint that may not be readily apparent on physical examination. Attention should also be given to soft tissue injuries that may have damaged the vascular supply. Damage to the dermis may result in sloughing tissue and exposure of the fracture. Crushing injuries of the digital vasculature and metacarpus can happen after forced fetal extraction during dystocia. Consideration should be given to sepsis at the fracture site, particularly in older fractures, as hematogenous spread of bacteria in the bovine can occur.

Cattle with fractures of the distal limb, forelimb, or hindlimb heal extremely well. Even comminuted and compound fractures can resolve if proper immobilization and therapy are provided. The most common method of repair in simple fractures is passive fixation with a cast. A half-limb cast is used for fractures below the carpus or tarsus.

Comminuted or compound fractures of the digits usually require a more rigid immobilization in order to get adequate healing in a reasonable amount of time. Of great value in complete immobilization is the traction/pin cast, which involves incorporation of wires passing through holes drilled in the rear abaxial walls of the hoof. This also serves to allow the assistant to properly position/align the limb for casting. Transfixation or transcortical pins are usually placed at some point in the lower to mid-third of metacarpal (MCIII) or metatarsal III (MTIII) to provide for weight transfer to the cast. Predrilling of a slightly smaller hole (0.5 m or less than the pin diameter) will reduce thermal injury to the bone during pin placement. Oftentimes, only one pin is needed to properly provide for weight transfer in younger animals. Threaded transcortical pin systems are preferred in older, heavier cattle, usually requiring placement of two pins at least 1.5 inches apart from each other. During casting, the pins are incorporated into the cast, with the pin ends cut even with the cast and protected by an acrylic compound placed over them, as well as acrylic applied to the bottom of

the cast to protect it. The wires placed in the hoof wall are incorporated into the wall of the cast under tension before completion of the procedure. Most of these casts can be removed at four to six weeks post casting.

Full Limb Casting

When dealing with fractures below the carpus/tarsus, it is always best served to apply a full limb cast. Single or multiple transcortical pins can be of value when incorporated with the cast as a complete fixation. This system provides complete immobilization and can even allow for "windows" to be placed in the casting to allow for local wound treatment in compound fractures. Single cortical fixators may be made with polyvinyl tubing. The one drawback to a large window in a cast is the local tissue edema that can occur at the site of tissue exposure at the opening. This can be controlled somewhat with pressure dressing at the wound site. The more complicated the fracture, the longer the cast may be required to stay in place. Be aware that young animals incurring extended time with a cast on are more predisposed to developing joint stiffness post removal. Fractured limbs that present with a large amount of edema should have the cast replaced 2–3 weeks after initial casting, at this time evaluating bone configuration and examining for any loose pins that may need to be replaced. Be aware if the animal's performance has been good in using the limb; if it becomes reluctant to use the casted limb there may be a problem with the transcortical device (pin fracture or osteomyelitis or both), which is commonly seen in the fourth to fifth week after application. If this is observed, removal of the pin should occur with lavage of the pin site after removal and possibly a course of antibiotics administered. This problem can specifically occur when too much heat is generated as the transcortical device is inserted in the bone. Occasionally a sequestrum may form within one or both cortices at the pin site, requiring aggressive curettage and lavage of the area. These areas will heal quickly. Once again, young animals heal rapidly and four weeks may be ample time for cast placement, yearlings usually require six weeks, and grown cattle require eight weeks of immobilization before cast removal.

Fractures of MCIII and MTIII respond very well to the use of transfixation pinning and casting or external skeleton fixation. In valuable individuals where cosmetic appearance is important, transfixation pinning/external skeletal fixation provides more complete immobilization and improved fracture healing, along with better overall alignment of the fracture fragments.

Special approaches are needed for fractures of the tibia and radius/ulna because external immobilization is difficult. Internal fixation with dynamic compression plates and screws should be considered in the extremely valuable individual, although clinical outcomes have not been very rewarding due to implant failure and postoperative sepsis. Success is more often seen with several different external coadaptations, along with skeletal fixators. In animals of lower genetic and economic value, the modified Thomas splint and a cast can be used to immobilize the limb. This system works well for fractures at the mid-shaft or the lower one-third of the affected bone and can be adapted as needed to allow for treatment of open wounds associated with the fractures. External skeletal fixation should be considered in the fractious individual since they seem prone to continually casting themselves on the splinted side, and therefore unable to rise on their own. This form of repair is especially desired when dealing with rough stock [19].

The Walker splint is another versatile splint device used for immobilization of the entire limb and is particularly valuable in tibial or radial fractures involving the proximal one-third of the bone. It is nearly always combined with casting of the distal limb. The design of the support structure as it relates to the groin area helps to protect the proximal limb from damage caused by abduction. It provides lateral support by cradling the gluteal muscles and keeping the stifle and upper limb more upright as opposed to being abducted. It is amazing how well calm cattle will handle these devices and heal the long bone fractures without additional coadaptation (Figures 16.27–16.29).

Fractures of the Proximal Limb

Fractures of the humerus usually occur after a fall or during a traumatic episode such as a bullfight or breeding injury. These fractures are best treated with stall rest,



Figure 16.27 Excellent treatment for transport of a comminuted fracture of MCIII. Notice a rebar stirrup support for the Robert Jones applied bandage. Stabilized fracture for an 800-mile ride.



Figure 16.28 Use of a pin cast and external fixators to stabilize a comminuted fracture of the Hereford heifer.



Figure 16.29 Thomas splint/cast and external fixators to immobilize severely comminuted fractures in a mature Longhorn steer.

supportive bandaging of the weight-bearing limb, and NSAIDs. Concurrent damage to the radial nerve can occur with fractures of the humerus, and supportive care with acupuncture and electrical stimulation has proven helpful. If tendon contracture of the affected limb starts to occur, the limb should be bandaged and splinted (Figure 16.30).



Figure 16.30 Fractured humerus with mild radial nerve involvement in a mature cow. Splintage of the lower leg was required to reduce deep digital flexor (DDF) contracture.

Surgical repair of femoral fractures using dynamic compression plates is not often successful in adults because of the difficulty in attaining alignment and compression with fixation screws, and only marginally successful in neonates. Salter-Harris fractures of the distal femur may be seen in calves delivered by forced extraction during dystocia. If attempted in the calf, it must receive appropriate colostrum supplementation and its health status should be thoroughly evaluated. Use of dynamic compression plates is difficult in neonates because the cortices of the femur are not strong enough to offer good purchase for the fixation screws. Stacked intramedullary pins or locking nail devices may be a better choice for the repair of the neonatal bovine [20].

Fracture Recovery

Immobilization times vary due to fracture configuration, age, and disposition of the animal. Young calves may heal a fracture within 3 weeks, whereas adult cattle with comminuted tibial fractures may require 10 weeks to heal. Radiographic evidence of fracture healing may not be

evident at the time of splint removal. A guideline to follow is to remove the splint if the limb feels stable and there is evidence some callus is being formed radiographically. Some form of continued splintage of the limb may be considered to lend support as active mineralization takes place. Weight transfer to the limb seems to increase mineralization of the callus, and within two weeks of removal, radiographic mineralization will be readily apparent. Activity after removal should be restricted for four to six weeks. Further time may be needed to completely rehabilitate the leg.

Hoof Trimming for Beef Cattle

Hoof trimming services are a great practice builder. Much of the literature available discusses hoof trimming in dairy cows, but the same basic principles can be applied to beef cattle as well. The Dutch five-step hoof trimming method is a good protocol to follow; however, any method of hoof trimming will certainly do as long as the animal has an even weight-bearing surface with proper hoof angles to ensure balance and symmetry of the hooves.

Most beef cattle presented for hoof trimming have grossly overgrown hooves, often due to issues with subclinical acidosis and multiple events of subclinical laminitis during their development. After restraint of the individual, either in a hoof-trimming chute or on a tilt table, the animal's hooves should be cleared of any excess organic debris. Evaluate the length and angulation of the hoof and identify the amount of wall and sole to be removed (Figure 16.31).

This process is almost automatic for the seasoned trimmer; less experienced practitioners should take their time and assess all four hooves before beginning the process.



Figure 16.31 Trimming the overgrown claw resulting from chronic subclinical laminitis.

Starting with the front hooves, use hoof nippers on the lateral claw to shorten the toe to 3.25–3.5 inches (Figure 16.32).

Proceed to trim the sole, leaving approximately 0.25 inches of sole; when the white line is apparent, you have removed enough sole. As sole is removed, make sure that the remaining sole surface is level and even, front to back. Next, the medial claw is shortened, and the solar surface is trimmed



Figure 16.32 First step in trimming of the hoof is shortening the claw to an acceptable length.



Figure 16.33 Completed trimming of a grossly overgrown hoof.

to match the lateral claw. When complete, both toes should be evaluated with a flat surface (a small board works well) to ensure that both hooves are level [21] (Figure 16.33).

Lastly, the sole should be modeled, scooping the axial portion of the claw to create a dish-like effect on the sole, which reduces the opportunity for mud or manure to build up between the toes and gives a more natural appearance to the hoof. After the claws have been properly shaped, address any solar defects and questionable wall areas.

Once the front hooves have been taken care of, the rear claws can be assessed and approached in reverse order, starting with the medial claw. Completion of the procedure should be followed by overall visualization of all four hooves, paying particular attention to the interdigital space. If any evidence of disease is observed in this region, it should be addressed before removing the animal from restraints. The animal should also be examined as it leaves the trimming area to ensure no lameness has developed.

Summary

Lameness evaluation and treatment can be a very rewarding portion of beef cattle practice. Economics sometimes play a role in what services can be provided, and the majority of patients may be limited to the economically valuable herd sires. At the end of the day, we have the opportunity to advance diagnostics and provide treatment options that will fit any scale of production, we just have to get past the mindset that it is "just a cow."

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Reproductive Surgery

17

Regional Anesthesia for Urogenital Procedures

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Introduction

When performing urogenital surgery in cattle, local and regional anesthesia are routinely utilized. They are a safe and effective alternative to general anesthesia in the large animal patient. Some benefits to using local and regional anesthesia include the need for minimal supplies to administer the necessary medications (anesthetic of choice, needles, and syringes), decreased risk of toxic effects, and minimizing bloat, regurgitation, and temporary or permanent nerve or muscle damage associated with positioning animals in recumbency. Many of the urogenital surgeries can be performed safely, rapidly, and humanely using physical restraint, sedation, and local or regional anesthesia. These techniques are easy to perform, the drug cost is minimal, and the effects are temporary and fairly well localized to the site of administration. This eliminates the widespread effects of general anesthesia on the entire body. The most common techniques used in bovine reproduction include infiltration anesthesia, nerve block anesthesia, and epidural anesthesia (Table 17.1).

Local Anesthetics

Many local or regional anesthetic drugs are available that can produce reversible loss of autonomic, motor, and sensory function with acceptable onset times and predictable duration [1]. These drugs vary in their potency, toxicity, and cost [2]. Lidocaine hydrochloride 2% and mepivacaine hydrochloride 2% have become two of the most widely used local anesthetic agents in cattle due to limited toxicity and low cost. Lidocaine hydrochloride has a duration of 90–180 minutes, is three times more potent than procaine, and diffuses into tissues more widely [1, 3]. Mepivacaine has a duration of 120–180 minutes, which is longer than lidocaine; however, the time to onset of anesthesia is similar. Although, bupivacaine has an even longer duration of action (up to 360 minutes), it is not recommended for routine use in cattle due to risk of inadvertent intravenous administration which could lead to toxic effects [4].

The addition of a vasoconstrictor such as epinephrine (5 μg/ml) to the local anesthetic solution (0.1 ml epinephrine 1 in 1000 added to 20 ml of local anesthetic) increases the potency and duration of activity of both regional and epidural anesthesia. However, local anesthetics containing epinephrine 1 in 200 000 should not be used in wound edges or in the subarachnoid space due to the risk of causing tissue necrosis and spinal cord ischemia [1].

Anesthesia for Laparotomy

Anesthesia of the paralumbar fossa and abdominal wall can be achieved by several techniques, including infusion of the incision or line block, the inverted L block, the proximal paravertebral nerve block, and the distal paravertebral nerve block. These anesthetic techniques are commonly used for reproductive procedures such as cesarean section, ovariectomy, and cervicopexy.

Line Block

Infusion of local anesthetic into the incision site or a line block may be used to desensitize a selected area of the paralumbar fossa. An 18-gauge, 3.8-cm needle is used to infuse multiple injections of 10 ml of local anesthetic solution subcutaneously and into the deep muscle layers and peritoneum. Pain of successive injections may be alleviated by placing the edge of the needle into the edge of the previously desensitized area at an angle of approximately 20–30° [2]. In heavily muscled or overweight cattle, it may be necessary to

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 17.1 Common nerve blocks with landmarks and volume of lidocaine for reference.

Nerve block	Landmark(s)	Volume of lidocaine
Proximal paravertebral	T13, L1, L2	6–8 ml ventral and dorsal to the transverse processes
Distal paravertebral	L1, L2, L4	10 ml dorsal and ventral to the transverse processes
Caudal epidural	S5–Co1, Co1–Co2	0.5 ml /45 kg
High-volume caudal epidural	S5–Co1, Co1–Co2	1 ml/5 kg
Sacral paravertebral	S3, S4, S5	2–3 ml per foramina
Internal pudendal	Lesser sacrosciatic foramen	20–75 ml per side
Castration	Skin and testicle	5–10 ml subcutaneous; 10–15 ml/200 kg into testicle

use an 18-gauge, 7.5-cm needle to penetrate through the large amount of subcutaneous fat to reach the deep muscle layers. The amount of local anesthetic needed to acquire adequate anesthesia depends on the size of the area to be desensitized. Adult cattle weighing 450 kg can safely tolerate 250 ml of a 2% lidocaine hydrochloride solution [2]. Delayed healing of the incision site is a possible complication of infiltration of local anesthetic at the surgical site.

Inverted L

The inverted L block is a non-specific regional block that locally blocks the tissue bordering the caudal aspect of the thirteenth rib and the ventral aspect of the transverse processes of the lumbar vertebrae [5]. An 18-gauge, 3.8-cm needle is used to inject up to a total of 100 ml of local anesthetic solution in multiple small injection sites into the tissues bordering the dorsocaudal aspect of the thirteenth rib and ventrolateral aspect of the transverse processes of the lumbar vertebrae (Figure 17.1). This creates an area of anesthesia under the inverted L block. Advantages of the inverted L block are that it is simple to perform, it does not interfere with ambulation, and deposition of anesthetic away from the incision site minimizes incisional edema and hematoma [1]. Disadvantages include incomplete analgesia and muscle relaxation of the deeper layers of the abdominal wall (particularly in obese animals), possible toxicity after larger doses of anesthetic, and increased cost because of larger doses of local anesthetic [1].

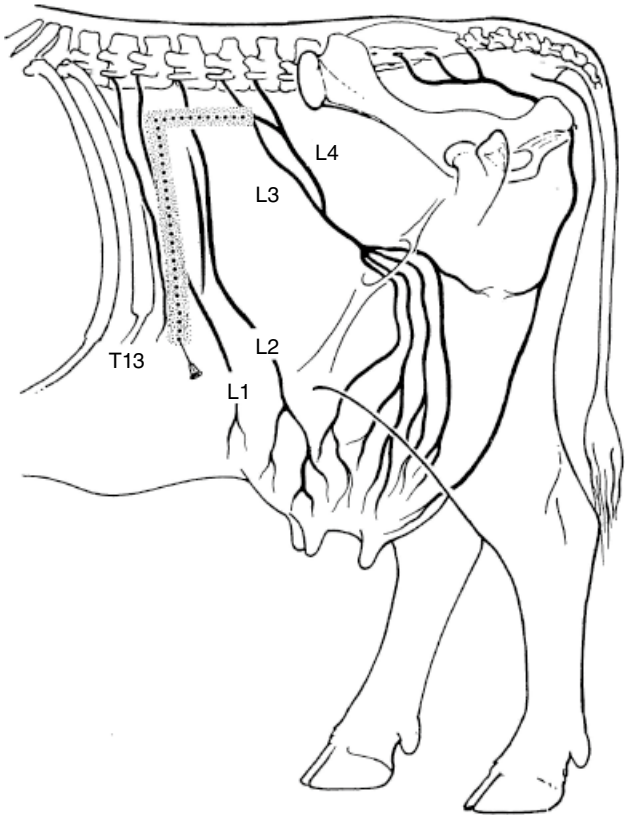


Figure 17.1 Inverted L. Ventral branches of labeled T13 and L1 to L4 are desensitized by this block. T13, Last thoracic nerve; L1–4, first to fourth lumbar spinal nerves. *Source:* From [1], © 2007, John Wiley & Sons.

Proximal Paravertebral

The proximal paravertebral nerve block desensitizes the dorsal and ventral nerve roots of the last thoracic (T13) and first and second lumbar (L1 and L2) spinal nerves as they emerge from the intervertebral foramina. To facilitate proper needle placement of anesthetic, the skin at the cranial edges of the transverse processes of L1, L2, and L3 and at a point 2.5–5 cm off the dorsal midline is desensitized by injecting 2–3 ml of local anesthetic using an 18-gauge, 2.5-cm needle. A 14-gauge, 2.5-cm needle is used as a cannula or guide needle to minimize skin resistance during insertion of an 18-gauge, 10- to 15-cm spinal needle. Approximately 5 ml of local anesthetic is placed through the cannula to anesthetize the needle tract for further needle placement.

To desensitize T13, the cannula needle is placed through the skin at the anterior edge of the transverse process of L1

at approximately 4–5 cm lateral to the dorsal midline. The 18-gauge, 10- to 15-cm spinal needle is passed ventrally until it contacts the transverse process of L1. The needle is then walked off the cranial edge of the transverse process of L1 and advanced approximately 1 cm to pass slightly ventral to the process and into the intertransverse ligament. A total of 6–8 ml of local anesthetic is injected with little resistance to desensitize the ventral branch of T13. The needle is then withdrawn 1–2.5 cm above the fascia or just dorsal to the transverse process and 6–8 ml of local anesthetic is infused to desensitize the dorsal branch of the nerve.

To desensitize L1 and L2, the needle is inserted just caudal to the transverse processes of L1 and L2. The needle is walked off the caudal edges of the transverse processes of L1 and L2, at a depth similar to the injection site for T13, and advanced approximately 1 cm to pass slightly ventral to the process and into the intertransverse ligament. A total of 6–8 ml of local anesthetic is injected with little resistance to desensitize the ventral branches of the nerves. The needle is then withdrawn 1–2.5 cm above the fascia or just dorsal to the transverse processes and 6–8 ml of local anesthetic is infused to desensitize the dorsal branch of the nerves (Figure 17.2).

Evidence of a successful proximal paravertebral nerve block includes increased temperature of the skin; analgesia of the skin, muscles, and peritoneum of the abdominal wall of the paralumbar fossa; and scoliosis of the spine toward the desensitized side. Advantages of the proximal paravertebral nerve block include small doses of anesthetic, wide and uniform area of analgesia and muscle relaxation, decreased intra-abdominal pressure, and absence of local anesthetic at the margins of the surgical site. Disadvantages of the proximal paravertebral nerve block include scoliosis of the spine, which may make closure of the incision more difficult, difficulty in identifying landmarks in obese and heavily muscled animals, and more skill or practice required for consistent results [2, 3, 5].

Distal Paravertebral

The distal paravertebral nerve block desensitizes the dorsal and ventral rami of the spinal nerves T13, L1, and L2 at the distal ends of the transverse processes of L1, L2, and L4, respectively. An 18-gauge, 3.5- to 5.5-cm needle is inserted ventral to the transverse process and 10 ml of local anesthetic is infused in a fan-shaped pattern. The needle can then be removed completely and reinserted or redirected dorsal to the transverse process, in a caudal direction, where 10 ml of local anesthetic is again infused in a fan-shaped pattern. This procedure is repeated for the transverse processes of the second and fourth lumbar vertebrae (Figure 17.3). Advantages of the distal paravertebral nerve block compared with the proximal paravertebral nerve block include lack of scoliosis, easier performance, and more consistent results. Disadvantages of the distal paravertebral nerve block compared with the proximal paravertebral nerve block include larger doses of anesthetic and variations in efficiency caused by differences in anatomic pathways of the nerves [2, 3, 5].

Anesthesia of the Perineum

Anesthesia of the perineum is routinely performed for obstetric procedures and for relief of rectal tenesmus in the cow. Many urogenital surgeries in cows (i.e. replacement

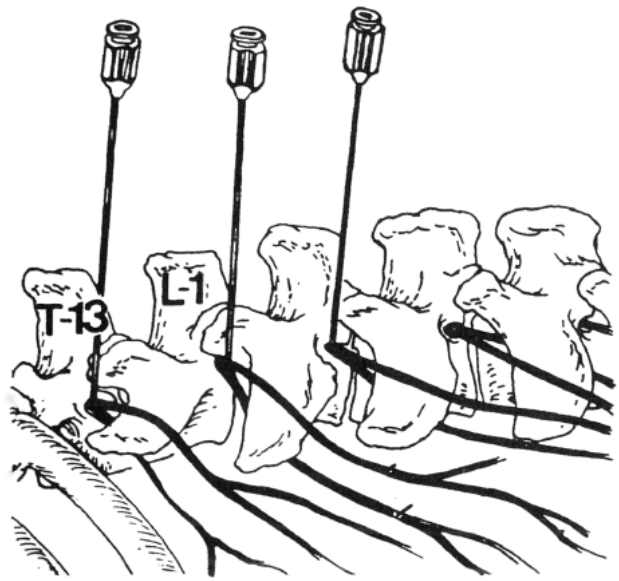


Figure 17.2 Proximal paravertebral. *Source:* From [2], © 1986, Elsevier.

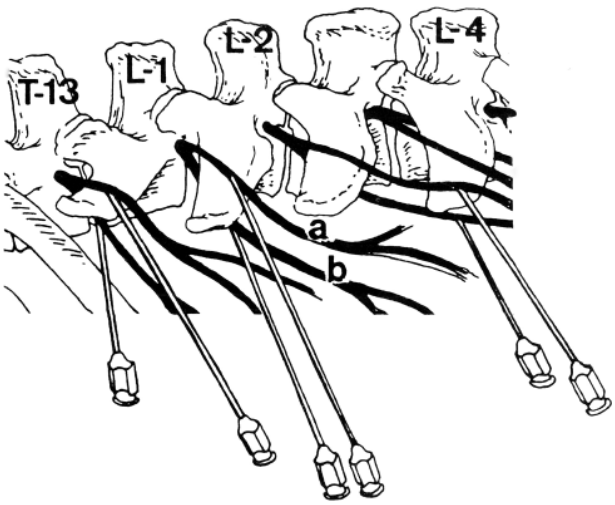


Figure 17.3 Distal paravertebral. *Source:* From [2], © 1986, Elsevier.

of vaginal/cervical prolapse) and bulls/steers (ischial urethrostomy, penile amputation) also require anesthesia of the perineum.

Caudal Epidural Anesthesia

Caudal epidural anesthesia is an easy and inexpensive method of analgesia that is commonly used in cattle. A high caudal epidural at the sacrococcygeal space (S5–Co1) desensitizes sacral nerves S2, S3, S4, and S5. The low caudal epidural at first coccygeal space (Co1–Co2) desensitizes sacral nerves S3, S4, and S5; as the anesthetic dose increases, nerves cranial to S2 may also become affected [6]. If possible, the hair should be clipped and the skin scrubbed and disinfected.

Standing alongside the cow, the tail should be moved up and down to locate the fossa between the last sacral vertebra and the first coccygeal vertebra (first freely moveable space) or between the first and second coccygeal vertebrae. An 18-gauge, 3.8-cm needle (with no syringe attached) is directed perpendicular to the skin surface. Once the skin is penetrated, place a drop of local anesthetic solution in the hub of the needle (hanging drop technique). The needle should then be advanced slowly until the anesthetic solution is drawn into the epidural space by negative pressure. The syringe may then be attached to the needle and anesthetic solution slowly injected with no resistance (Figure 17.4a). The dose of local anesthetic to be used is 0.5 ml per 45 kg body weight.

An increased dose of local anesthetic can be used to facilitate other urogenital procedures including teaser bull preparation and ventral midline cesarean section. This is referred to as high-volume caudal epidural anesthesia and is performed in the same way as the standard caudal epidural, only increasing the volume of local anesthetic used. The recommended dose is 1 ml per 5 kg body weight; however, volumes up to 0.5 ml/kg have been used without adverse effects. The patient will lose control of the hindlimbs, so recumbency is a must when using this technique. The effects on the hindlimbs may last up to four to six hours after administration. It is recommended that hobbles be placed on the hindlimbs and the patient be recovered in an area with good footing and left alone until they are able to stand unassisted [4, 7].

Continuous Caudal Epidural Anesthesia

Continuous caudal epidural anesthesia is used in cattle with chronic rectal and vaginal prolapse that experience continuous straining after the initial epidural. This procedure is performed by placing a catheter into the epidural space for intermittent administration of local anesthetic. A 17-gauge, 5-cm spinal needle (Tuohy needle) with stylet in place is inserted into the epidural space at Co1 to Co2 with the bevel directed craniad. The stylet is removed, and 2 ml of local anesthetic is injected to determine if the needle is in the epidural space. A catheter is inserted into the needle and advanced cranially for 2–4 cm beyond the needle tip. The needle is then withdrawn while the catheter remains in place (Figure 17.4b). An adapter is placed on the end of the catheter and the catheter secured to the skin on the dorsum. Local anesthetic solution may then be administered as needed [2].

Additives or Alternatives to Local Anesthetic Epidural Anesthesia

More recently, α_2-adrenergic agonists and opioids either alone or in combination with local anesthetic solutions have been used for epidural anesthesia. Epidural administration of the

(a) (b)

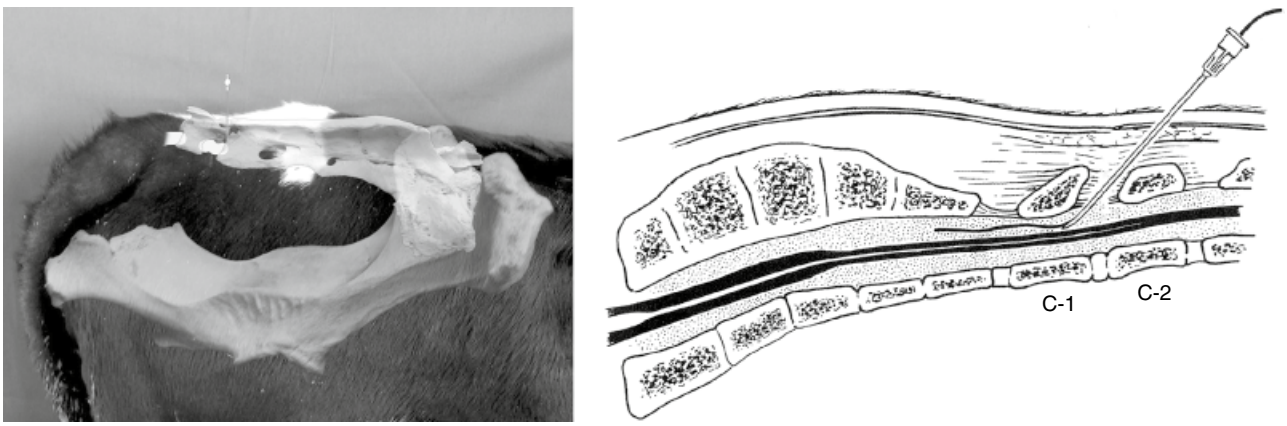


Figure 17.4 (a) Needle placement for caudal epidural. *Source:* Image courtesy of Dr. Douglas Hostetler. Adapted with permission from *Lumb and Jones' Veterinary Anesthesia*. (b) Catheter placement for continuous flow epidural. *Source:* From [1], © 2007, John Wiley & Sons. Adapted with permission from *Lumb and Jones' Veterinary Anesthesia*.

α_2-agonist xylazine hydrochloride (0.05 mg/kg) diluted in 5–12 ml of sterile saline or xylazine hydrochloride (0.3 mg/kg) added to 5 ml of 2% lidocaine hydrochloride offer similar anesthesia to lidocaine. Although the duration of anesthesia is prolonged (four to five hours) using these combinations, systemic effects (sedation, salivation, ataxia) may also occur [1]. Epidural administration of opioids, such as morphine (0.1 mg/kg) diluted in 20 ml of sterile saline, is used to provide analgesia for a prolonged period (approximately 12 hours) without interfering with motor function. Disadvantages of using opioids for epidural anesthesia are that the analgesia is not as potent as lidocaine and the maximum effect of a morphine epidural may not occur for two to three hours. Caudal epidural administration of morphine may be used to help alleviate pain in the perineal area and straining [8].

Alcohol has been used previously to desensitize the perineum for a prolonged period and in some cases permanently. Alcohol administered by this route can lead to demyelination of nerves. This can result in damage more cranial than the perineum and could effectively paralyze the patient if it were to travel up the nerve root and into the sciatic nerve. Therefore its use in epidural anesthesia is not recommended [9].

Sacral Paravertebral Anesthesia

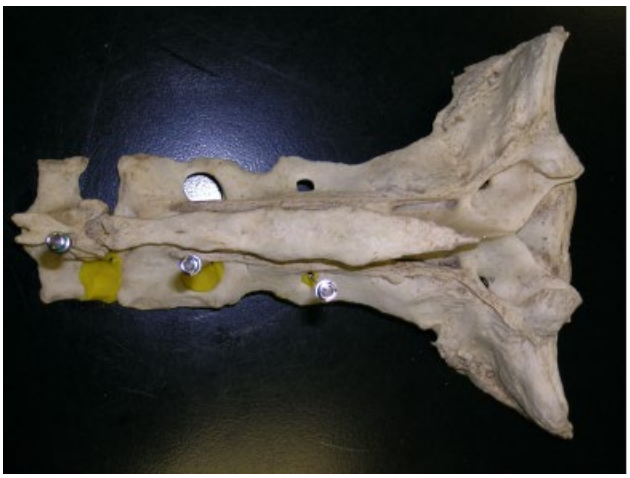
Sacral paravertebral anesthesia is used to relieve rectal tenesmus associated with rectal prolapse without affecting the sciatic nerve and function of the tail or the animal's ability to stand. The sacral paravertebral nerve block is used to provide analgesia to the pudendal nerve (pudic nerve), medial hemorrhoidal nerve (pelvic splanchnic nerve), and caudal hemorrhoidal nerve (caudal rectal nerve) by blocking S3, S4, and S5 as they branch off the spinal cord, thereby providing analgesia to the anus, vulva, and vagina [1, 10]. In bulls, S3 supplies motor function to the retractor penis muscles. Physical restraint in a squeeze chute and/or sedation may be beneficial in order to prevent lateral movement of the animal during the procedure. In addition, a caudal epidural may be helpful if the animal is fractious. The skin over the dorsal sacrum should be clipped of hair and surgically prepared for the procedure. The paired S5 foramina are 1–2 cm lateral to the sacral coccygeal joint. The S4 foramina are about 3–4 cm cranial and more lateral to the S5 foramina. The S3 foramina are an additional 3–4 cm cranial to the S4 foramina (Figure 17.5a and b). A stab incision can be made dorsal to each foramen to aid in the introduction of an 18-gauge, 5- to 7-cm needle. The foramina can be palpated rectally with a finger placed in or over the ring which allows for identification of the foramen and ensures correct needle placement (Figure 17.6). Once the needle has entered the osseous ring, inject 2–3 ml of lidocaine hydrochloride; this should be repeated for each foramen [10]. The use of a lidocaine/alcohol mixture has also been described to manage tenesmus following chronic cervicovaginal prolapse or rectal prolapse. A mixture of 1 ml of 2% lidocaine hydrochloride and 2 ml of 95% ethyl alcohol has been used effectively [10].

Anesthesia of the Penis and Prepuce

Desensitization of the Internal Pudendal Nerve Block

The procedure for bilateral internal pudendal (pudic) nerve block was first described by Larson [11] to facilitate relaxation of the bull's penis without causing locomotor impairment.

(a)



(b)

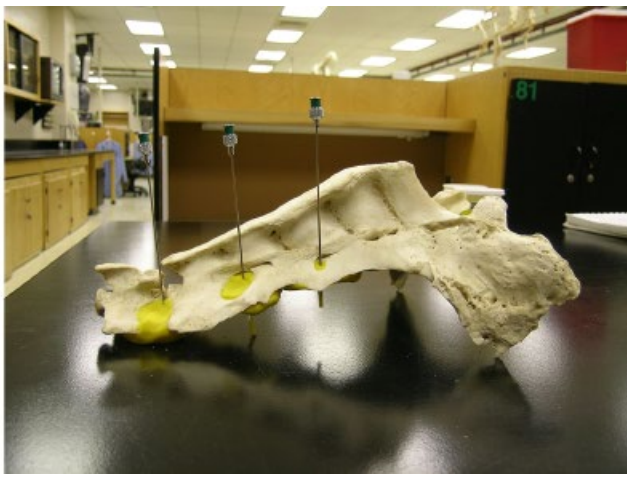


Figure 17.5 Dorsal view (a) and lateral view (b) of S3, S4, and S5 foramina with needle placement. *Source:* Image courtesy of Douglas Hostetler.

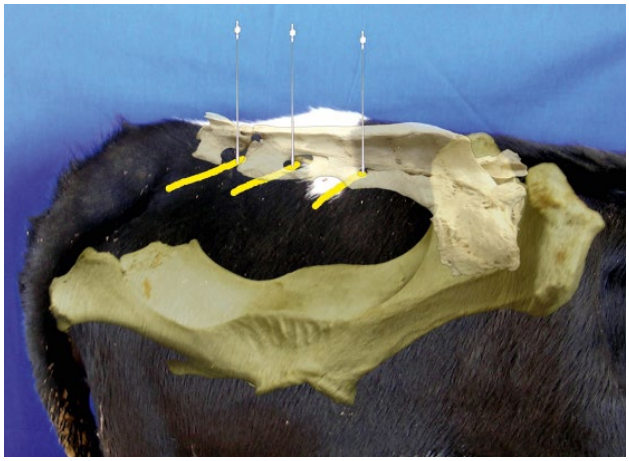


Figure 17.6 Sacral paravertebral. *Source:* Image courtesy of Douglas Hostetler.



Figure 17.7 Injection of 2 ml local anesthetic in the skin at the ischiorectal fossa.



Figure 17.8 Needle placement for pudendal nerve block. *Source:* Image courtesy of Douglas Hostetler.

The internal pudendal nerve block can be used in the standing bull for penile relaxation and analgesia distal to the sigmoid flexure and examination of the penis. In the standing female, the internal pudendal nerve block can be used to relieve straining caused by chronic vaginal prolapse. This technique may also be used for surgical procedures of the penis, such as repair of prolapses, removal of peripenile tumors, removal of penile papillomas or warts, and other minor surgeries of the penis and prepuce.

This procedure involves desensitizing the internal pudendal nerve and the anastomotic branch of the middle hemorrhoidal nerve using an ischiorectal approach. The internal pudendal nerve consists of fibers originating from the ventral branches of the third and fourth sacral nerves (S3 and S4) and the pelvic splanchnic nerves. The skin at the ischiorectal fossa on both sides is clipped, disinfected, and desensitized with approximately 2 ml of local anesthetic (Figure 17.7). A 14-gauge, 1.25-cm needle is inserted through the desensitized skin at the ischiorectal fossa to serve as a cannula. An 18-gauge, 10- to 15-cm spinal needle is then directed through the cannula to the pudendal nerve. The operator's left hand is placed into the rectum to the level of the wrist and the fingers directed laterally and ventrally to identify the lesser sacrosciatic foramen. The lesser sciatic foramen is first identified by rectal palpation as a soft depression in the sacrosciatic ligament. The internal pudendal nerve can be readily identified lying on the ligament immediately cranial and dorsal to the foramen and approximately one finger's width dorsal to the pudendal artery passing through the foramen. The internal pudendal artery can be readily palpated a finger's width ventral to the nerve. The spinal needle is held in the operator's right hand and introduced through the cannula in the ischiorectal fossa. The spinal needle is directed medial to the sacrosciatic ligament and directed cranioventrally (Figure 17.8). The needle is not felt until it has been introduced approximately 5–7 cm and can then be repositioned to the nerve. Once at the pudendal nerve, 20–75 ml of local anesthetic is deposited at the nerve (Figure 17.9). The needle is then removed and the site of deposition is massaged to aid in dispersal of the local anesthetic. This procedure is repeated on the opposite side of the pelvis. Relaxation of the penis varies and may take as long as 30–40 minutes for full effect. Effectiveness of the block can be assessed by firmly squeezing the tail of the epididymis of each testicle. The bull's inability to lift or retract the testicle signifies adequate analgesia. The duration of the internal pudendal nerve block lasts two to four hours [10].

Desensitization of the Dorsal Nerve of the Penis

The dorsal nerve of the penis may be desensitized at a location just proximal to the surgical site. With the bull restrained, the penis should be manually extended and a towel clamp should then be placed under the dorsal apical ligament.



Figure 17.9 Pudendal nerve block.

Alternatively, a gauze tourniquet may be placed around the free portion of the penis to aid in penile extension. With the dorsal aspect of the penis thoroughly cleansed, 2–4 ml of 2% lidocaine hydrochloride should be infused subcutaneously across the dorsum of the penis proximal to the lesion [9].

Alternatively, the dorsal nerve of the penis may be desensitized as it passes over the ischial arch for penile anesthesia and relaxation. The skin associated with the penile body and located 10 cm ventral to the anus and 2.5 cm from midline is infiltrated with 2–4 ml of 2% lidocaine hydrochloride using a small-gauge needle (22–25 gauge). A 20-gauge, 4-cm needle is then inserted through the desensitized skin and advanced for 5–7 cm to contact the pelvic floor. Aspiration ensures that the needle is not in the dorsal artery of the penis. The needle is then withdrawn approximately 1 cm and the area infiltrated with 20–30 ml of 2% lidocaine hydrochloride. The procedure is repeated on the opposite side of the penis. Analgesia and paralysis of the penis will occur within 20 minutes and should last for one to two hours [1].

Anesthesia for Castration

Castration of bulls is a very common surgical procedure in general practice. Historically, castration was often performed with minimal or no anesthesia. However, anesthesia for castration is more commonly practiced because calves benefit from anesthesia with improved feed consumption and rate of gain. Depending on the age and size of the animal, the surgery is usually performed with chemical and/or regional anesthesia (scrotum and testicles). Depending on the size of the calf, the proposed line of incision for removal of the distal aspect of the scrotum should be subcutaneously infiltrated with 5–10 ml of 2% lidocaine hydrochloride. In bulls, an 18-gauge, 3.8-cm needle is inserted at an angle (30–45°) into the center of the testicle and 10–15 ml of local anesthetic per 200 kg body weight is injected into the parenchyma of each testicle. The anesthetic quickly enters the lymphatics and desensitizes the sensory fibers in the spermatic cord. For smaller animals or calves, a smaller needle (20 gauge, 2.5 cm) may be used to administer 2–10 ml of 2% lidocaine hydrochloride [1].

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18

Surgery of the Scrotum and its Contents

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Introduction

The scrotum of the bull is a dependent appendage of the ventral abdominal skin that supports and protects the testicles and helps regulate testicular temperature. The testis must be maintained at a temperature 4–6 °C cooler than body temperature for normal spermatogenesis to occur. Testicular thermoregulation is a complex process that involves a combination of evaporative cooling from scrotal sweat glands, relaxation and contracture of the tunica dartos muscle in response to scrotal temperature, and counter-current heat exchange between the pampiniform plexus and the testicular artery [1]. Trauma, inflammation, or swelling of the scrotum or testes can easily impair testicular thermoregulation, and studies utilizing scrotal insulations have shown that morphological abnormal sperm are produced within 6–14 days of the insult [2].

Physical Examination of the Scrotum and Testicles

Carefully examine the scrotum for dermatitis, edema, scar tissue, and symmetry [3]. Palpate the testicles for relative size, firmness, symmetry, evidence of pain or swelling, presence of fluid in the vaginal cavity, and the ability of the testicles to move freely within the vaginal cavity. There should be no more than 10% difference in the size of the testes and normal testicular tone approximates that of liver.

Ultrasound of the Testicles

Diagnosis of scrotal or testicular disease may be aided by B-mode real-time ultrasound using a 5-MHz probe. Normal testicles are homogeneous and moderately echogenic [4] (Figure 18.1). The mediastinum testis is a readily identifiable hyperechoic area in the center of the testicle when viewed in the transverse plane or a hyperechoic line when viewed in the sagittal plane (Figure 18.2). The head, body, and tail of the epididymis are less echogenic than the testicle and are readily identified as they course along the testicle. Thickness of the scrotal skin and vaginal tunics and the presence of fluid within the vaginal cavity are readily determined.

Thermography of the Scrotum

Testicular temperature must be below body temperature for normal spermatogenesis in bulls. Infrared thermography provides a non-invasive, non-contact imaging technique to determine normal and abnormal thermal patterns in bulls and other animals [5]. Thermography utilizes sensitive infrared imaging to produce a color photograph that portrays variations in surface temperature that reflect the temperature of tissues immediately beneath the skin. The normal thermogram of the scrotum in all species is a left and right symmetric pattern with a constant decrease in temperature gradient from the base to the apex (Figure 18.3). In bulls a temperature gradient of 4–6 °C from base to apex is considered normal. A gradual decrease from base to apex with concentric color

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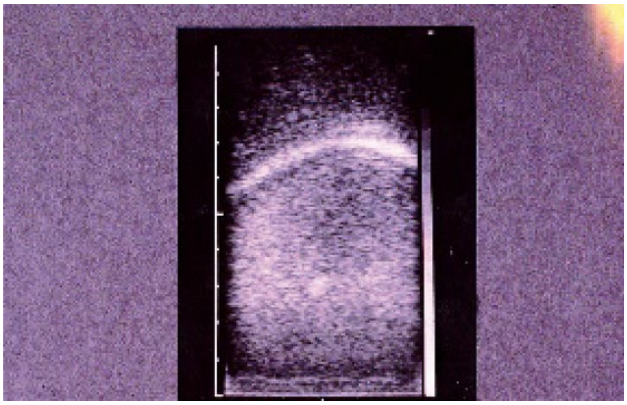


Figure 18.1 Ultrasound of normal testis in transverse plane. Mediastinum testis is hyperechoic area in center of testis.

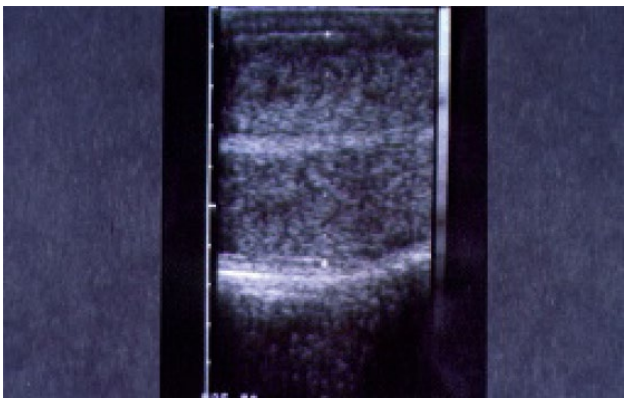


Figure 18.2 Sagittal ultrasound of normal bovine testicle. Mediastinum testis is hyperechoic line in center of testis.

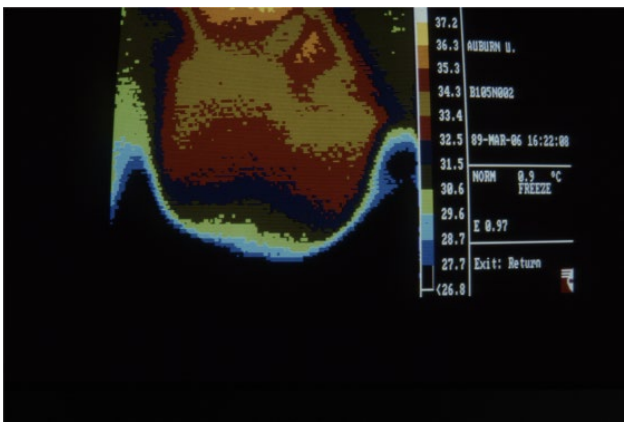


Figure 18.3 Normal thermographic pattern of bull scrotum.

bands is consistent with normal function of the vascular counter-current heat-exchange mechanism of the testes. An excessively cool or excessively warm area as evidenced by thermographic color is consistent with testicular disease or injury [6, 7] (Figure 18.4).

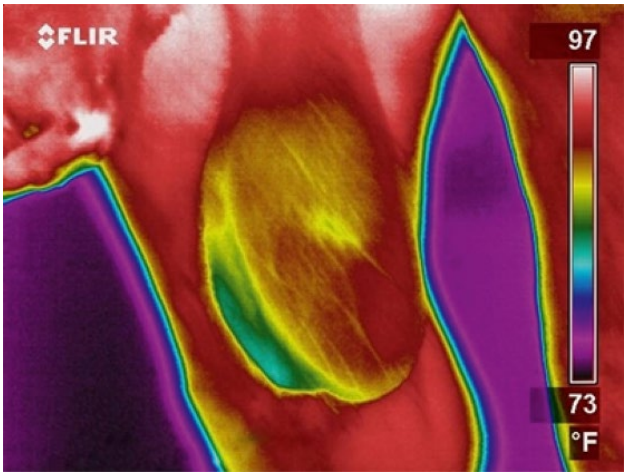


Figure 18.4 Thermograph of scrotum of bull with unilateral pathology. Note decreased temperature on left side of scrotum.

Semen Evaluation

Semen from bulls with significant scrotal or testicular pathology generally does not meet the minimum 70% morphologically normal sperm of which more than 30% are progressively motile, and these bulls are thus considered subfertile. The temporal appearance of sperm abnormalities following scrotal insulation has been established in bulls and may be useful in determining the chronicity of an insult [2, 8, 9]. Semen evaluation may also be used to determine when surgical intervention should be pursued in bulls with unilateral testicular disease.

Scrotal and Testicular Disease and Injury

Fluid Accumulation in the Vaginal Cavity

Fluid accumulation in the vaginal cavity is a common cause of scrotal enlargement in bulls. This accumulation can occur bilaterally, but unilateral enlargement is more common. The condition can be further defined by the type of fluid present. Hemorrhage in the vaginal cavity commonly results from trauma and is known as a hematocele. Injury or periochitis can cause the accumulation of an inflammatory exudate or transudate in the vaginal cavity which is referred to as a hydrocele [10]. Bilateral scrotal hydroceles have been reported in bulls secondary to ascites caused by gastrointestinal parasitism [11]. Fluid accumulation in the vaginal cavity causes distention of the parietal vaginal tunic, creating the appearance of testicular swelling. Depending on the nature of the fluid present and the degree of distension of the parietal

vaginal tunic, the affected side may palpate firm and the testicle itself may be hard to identify. The presence of fluid in the vaginal cavity can easily be confirmed with ultrasound (Figure 18.5). Fibrinous and fibrous adhesions between the testicle and the parietal tunic may also be visible on ultrasound.

Orchitis, Periorchitis, and Epididymitis

Orchitis is uncommon in bulls and usually unilateral. Infection is often subclinical but rarely may range to severe and perhaps suppurative [12, 13]. The route of infection is primarily hematogenous but penetrating wounds of the scrotum are also possible. The dense fibrous tunica albuginea surrounding the testicle limits this organ's potential for enlargement and therefore there is usually not significant testicular swelling. However, when infection involves the surrounding tunica vaginalis (periorhitis), significant scrotal enlargement can occur. The affected side may also be warm and painful to the touch, with pain significant enough to alter the gate of the bull [12].

Epididymitis occurs more commonly than orchitis in bulls and may be diagnosed by thorough examination of the testes and scrotal contents [14–16]. The condition is often unilateral in the bull and may present as a swollen painful epididymal tail in the acute phase of the disease. Chronic epididymitis usually results in epididymal tails that are small and firm, and infertility is often caused by their eventual obstruction.

Trauma

Trauma is a common cause of pathological changes in scrotal contents. As mentioned above, trauma often leads to the accumulation on fluid in the vaginal cavity in the form of a hematocele or hydrocele. Penetrating wounds of the scrotum can result in orchitis/periorchits with potential scrotal abscessation. Severe trauma can induce rupture of the tunica albuginea [10]. Leakage of sperm due to tubular damage of the testicle can invoke an autoimmune reaction leading to granulomatous reactions. Depending on the acuteness and severity of the trauma, significant scrotal enlargement and edema may be present (Figure 18.6).

Inguinal Hernia

Inguinal hernias can be classified as either congenital or acquired and direct or indirect. In mature bulls all inguinal hernias are considered acquired. Indirect hernias occur when bowel passes through an intact but dilated inguinal ring. This type of herniation occurs more frequently on the left side and rarely causes strangulation of bowel. Bulls with an indirect hernia will typically present clinically normal, other than swelling in the neck of the scrotum that gives the scrotum an hourglass appearance (Figure 18.7). Direct inguinal hernias occur through the inguinal ring, but disruption or tearing of the ring or vaginal tunic is present. Direct inguinal herniation is more likely to result in strangulation and obstruction of bowel with more pronounced and generalized scrotal swelling [17, 18].

The presence of an inguinal hernia can be confirmed with rectal palpation and scrotal ultrasound. Palpation should reveal a dilated or disrupted inguinal ring on the affected side and the presence of herniated bowel contained in the ring. Ultrasound of the neck of the scrotum can definitively confirm the presence of bowel. Bulls with intestinal obstruction will present with abdominal pain and distention and will require emergency surgery. Inguinal hernias in bulls that present clinically normal without any signs of obstruction can be managed with elective surgery [17, 18].

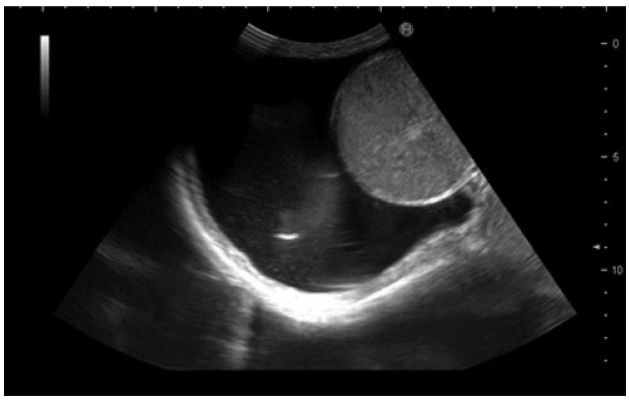


Figure 18.5 Ultrasound of scrotum of a bull with a hydrocele. Note anechoic fluid distention of vaginal cavity. *Source:* Image courtesy of Darcie Sidelinger and Heath King.



Figure 18.6 Bull with acute bilateral scrotal enlargement secondary to trauma. Hemicastration was performed to remove a ruptured testicle. *Source:* Image courtesy of Richard Hopper and Heath King.



Figure 18.7 Inguinal hernia in a bull. Note characteristic swelling confined to neck of scrotum.

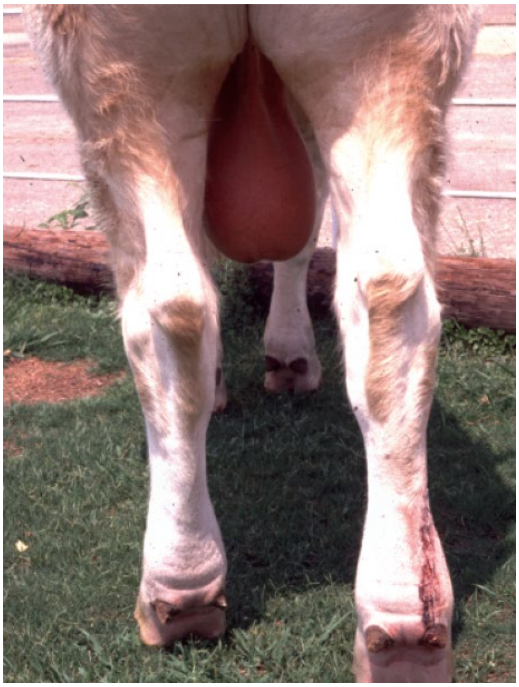


Figure 18.8 Generalized edema of scrotal wall due to *Mycoplasma weyenoii*.

Less Common Causes

Bilateral scrotal enlargement with pitting edema of the hind limbs has been reported in young bulls secondary to *Mycoplasma wenyonii* infection (Figure 18.8). Swelling subsided in these bulls following treatment with oxytetracycline [19, 20]. Scrotal edema can be differentiated from other causes of scrotal enlargement by palpation and ultrasound.

Primary testicular tumors are not common in bulls but unilateral or bilateral scrotal enlargement has been reported in bulls with mesothelioma [5, 12, 13, 19].

Unilateral Castration

Hemi- or unilateral castration should be considered in bulls with unilateral testicular disease. When one testicle is inflamed, the heat thus generated affects the contralateral testicle and the reversibility of degenerative change depends on the severity and duration of the insult [12]. Valuable animals with unilateral hematoceles, hyrdrocleles, orchitis, periorchitis, or testicular rupture may be able to resume breeding soundness with unilateral castration.

Although unilateral castration is not an emergency procedure, the prognosis for return to fertility improves if surgery is performed early in the disease process. Early intervention can reduce further insult to the contralateral testicle generated by inflammation, with less likelihood that the testicle will undergo irreversible degeneration. Most bulls will return to fertility following surgery if the remaining testicle is not severely compromised [10, 21, 22]. Compensatory hypertrophy in the normal testis should allow the bull to subsequently produce up to 75% of normal sperm capacity. Therefore, advise owners that restrictions may be necessary following surgery. Additionally, although a bull may be productive in a herd situation, a unilaterally castrated bull will not pass a standardized breeding soundness evaluation and is therefore ineligible for certain shows or sales [10].

In preparation for surgery the bull should be withheld from feed for 48 hours and water for 12 hours. Surgery is performed with the bull restrained in lateral recumbency under general anesthesia or heavy sedation and regional or local anesthesia. Regional anesthesia can be accomplished with the use of a pudendal nerve block, or local anesthetic can be injected into the neck of the scrotum and the spermatic cord. Antibiotics are administered prior to surgery and continued for at least five days post-surgery. Nonsteroidal anti-inflammatories should also be administered immediately prior to surgery. The scrotum is clipped and prepped for aseptic surgery [10, 23]. On the lateral aspect of the scrotum an elliptical skin incision is made approximately the length of the testicle, preserving the parietal vaginal tunic (Figure 18.9). The affected testicle within the parietal vaginal tunic is bluntly dissected away from the surrounding scrotal fascia (Figure 18.10). If the condition is chronic, variable amounts of fibrous tissue and adhesions of the parietal and visceral vaginal tunics may be

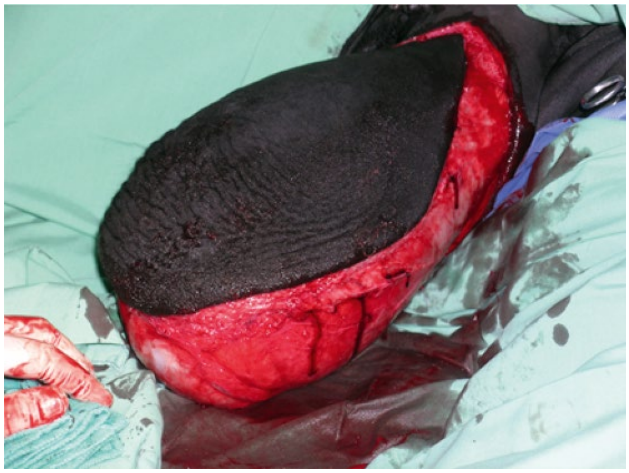


Figure 18.9 Elliptical skin incision on lateral aspect of scrotum. *Source:* Image courtesy of Richard Hopper and Heath King.

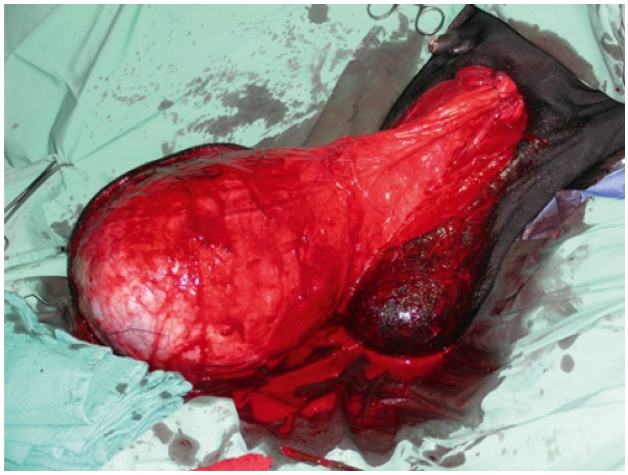


Figure 18.10 Testicle within parietal vaginal tunic bluntly dissected from scrotal fascia. *Source:* Image courtesy of Richard Hopper and Heath King.

encountered. At this point the decision is made whether an open or closed castration should be pursued. An open castration allows for more careful ligation of the vasculature but does expose the surgery site to potential contamination from the contents of the parietal tunic. A closed castration is typically performed by the author to limit contamination of the surgical site. Both techniques are described below.

To perform an open castration, a vertical incision is made through the parietal tunic sufficiently long to remove the testis and exteriorize the testicle and spermatic cord (Figures 18.11). The spermatic artery, vein, and ductus deferens are isolated and double ligated with #0 chromic gut 8 cm proximal to the pampiniform plexus and transected between the two ligatures (Figure 18.12). The vaginal tunic is then transected and the external cremaster muscle is ligated and transected distal to the stump of the spermatic

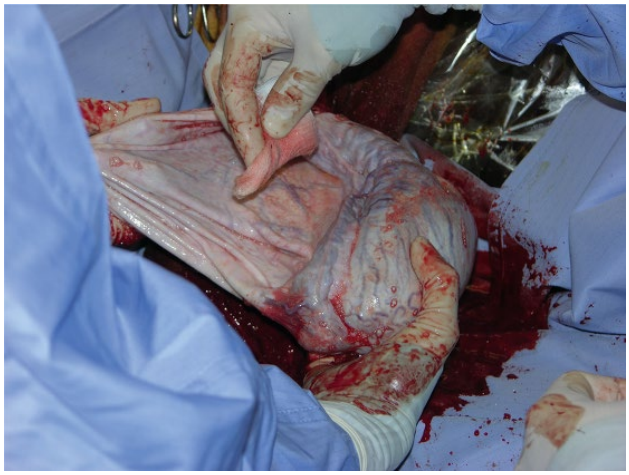


Figure 18.11 Parietal vaginal tunic incised and testicle exposed. *Source:* Image courtesy Darcie Sidelinger & Heath King.

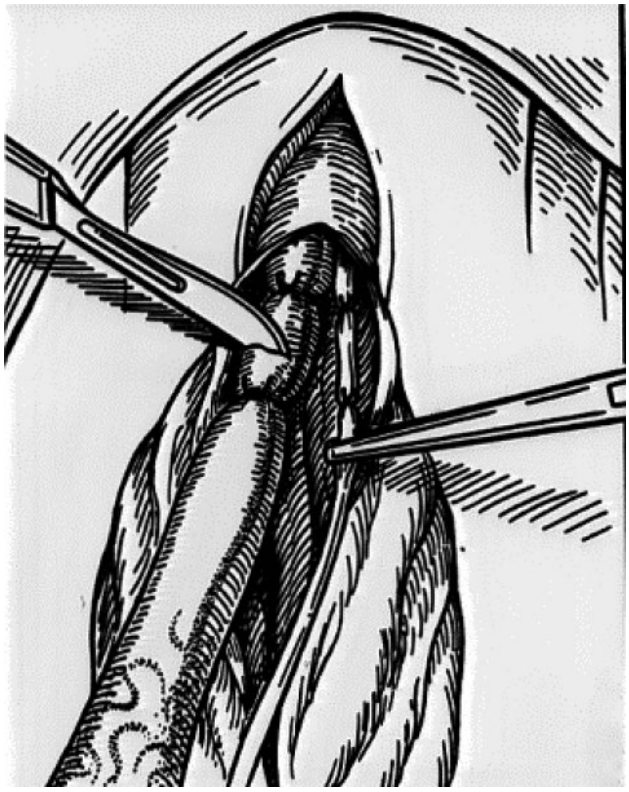


Figure 18.12 Schematic of double ligation and transection of spermatic cord and vessels.

cord (Figure 18.13). The vaginal tunic is closed with an inverting pattern such as a Connell or Parker–Kerr using #0 chromic gut (Figure 18.14). To perform the closed castration, the spermatic cord is ligated proximal to the pampiniform plexus with a combination of one transfixing ligature and two circumferential Miller's knot ligatures using #4 polyglactin suture (Figure 18.15). The spermatic cord is then transected distal to the ligatures with emasculators.



Figure 18.13 Transected stump of vaginal tunic following inverting suture closure. *Source:* Image courtesy Darcie Sidelinger & Heath King.

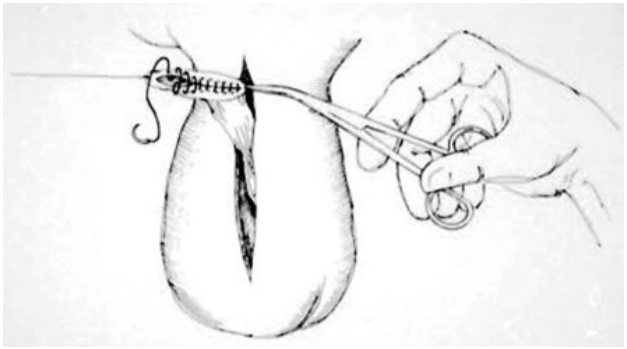


Figure 18.14 Schematic of inverting closure of vaginal tunic.

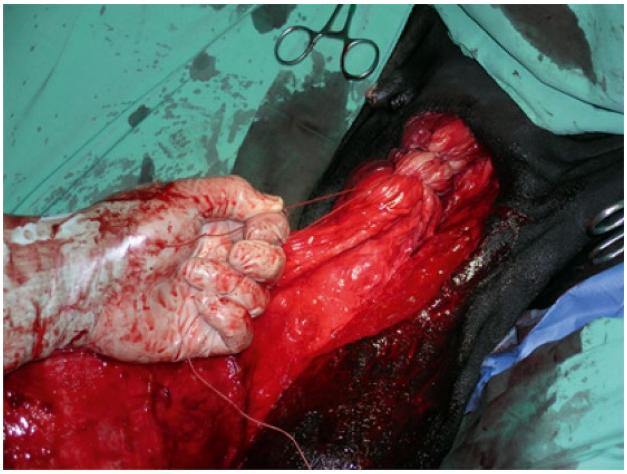


Figure 18.15 Ligation of spermatic cord for a closed castration. *Source:* Image courtesy of Richard Hopper and Heath King.

For either technique, the scrotal fascia and tunica dartos are closed with #0 or larger chromic gut in a simple continuous pattern being careful to eliminate dead space. The skin is then closed with non-absorbable suture in a continuous pattern of the surgeon's choice (Figure 18.16).

Postoperative swelling can be reduced by wrapping the scrotum in 4 inches of elastic adhesive tape (Figure 18.17). The bandage should be removed in 24–48 hours. Skin sutures are removed in 10–14 days and the bull can return to service when the spermiogram returns to normal. Normal bulls undergoing unilateral orchiectomy had normal spermiograms by 14 days after surgery [16]. Bulls with unilateral scrotal pathology and transient testicular degeneration of the remaining testicle should be expected to return to normal sperm production approximately 60 days after removal of the diseased or injured testicle [17].



Figure 18.16 Scrotal skin incision closed with continuous interlocking pattern. *Source:* Image courtesy of Richard Hopper and Heath King.



Figure 18.17 Postoperative bandaging of scrotum to minimize swelling. *Source:* Image courtesy of Richard Hopper.

However, if the surgery is performed during the warmer months of the year, normal testicular function may not resume until ambient temperatures moderate.

Inguinal Hernia Repair

The two approaches for inguinal hernia repair in bulls are the standing flank approach and the inguinal approach performed in lateral recumbency. The primary advantage of the flank approach is that it does not require general anesthesia. However, not all hernias can be reduced through the flank approach and closure of the inguinal ring must be performed blindly. Utilizing the inguinal approach requires general anesthesia but provides much better surgical exposure and simplifies closure of the inguinal ring. The improved exposure allows for the identification and transection of adhesions between the herniated bowel and parietal tunic and facilitates resection and anastomosis of injured and strangulated bowel. Therefore bulls that present with an inguinal hernia and evidence of abdominal pain should be repaired by the inguinal approach [18].

Standing Flank Approach

The ipsilateral paralumbar fossa is clipped and prepared for aseptic surgery. An inverted L or paralumbar block is used for local anesthesia. The abdomen is then entered in a routing manner and the inguinal ring is identified. The herniated bowel is grasped and traction is applied to reduce the hernia (Figure 18.18). Failure to reduce the hernia by traction at this stage will require transition to the inguinal approach described below. Once the hernia is reduced, closure of the inguinal ring is performed by blindly placing several interrupted sutures or by utilizing the continuous blind suture technique described in Chapter 55. Care must be taken during closure of the inguinal ring to avoid the vasculature entering and exiting the abdomen through the ring. The flank is then closed in a routing manner [17, 18].

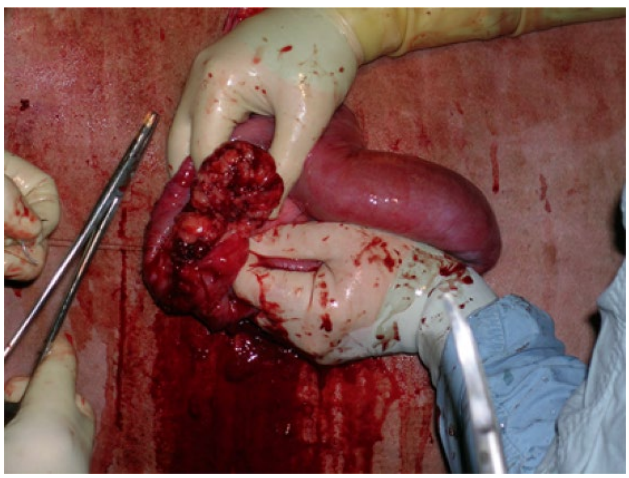


Figure 18.18 Resection and anastomosis of herniated bowel removed from inguinal ring by flank approach. *Source:* Image courtesy of Richard Hopper.

Inguinal Approach

For the inguinal approach, the bull is anesthetized and placed in lateral recumbency with the affected side up. The upper rear leg must be elevated to expose the inguinal area. The inguinal area is clipped and prepared for aseptic surgery. A 20- to 25-cm incision is made over the inguinal ring (Figure 18.19). The subcutaneous tissues are bluntly and sharply dissected to expose the spermatic cord. An incision is then made in the parietal tunic to expose the herniated tissues (Figure 18.20). The herniated bowel is examined for adhesions or evidence of strangulation. If encountered, strangulated or devitalized bowel should be removed by resection and anastomosis. Adhesions between the bowel and parietal tunic should be ligated before separation [17, 18]. The herniated bowel is then reduced. The decision is made as to whether the testicle on the affected side will be spared or removed. Removing the testicle will prevent postoperative complication that could impact testicular thermoregulation and allows for simplified and complete closure of the inguinal ring.

Closure of the inguinal ring, when the testicle is spared, is accomplished in two stages. The first stage pulls the internal



Figure 18.19 Incision over inguinal ring. *Source:* Image courtesy of Darcie Sidelinger and Heath King.

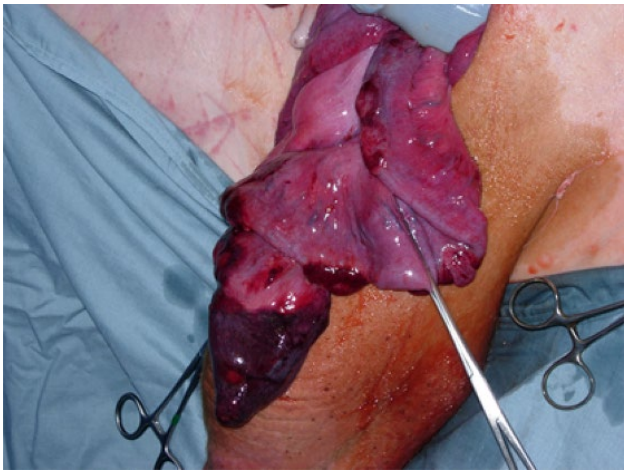


Figure 18.20 Herniated bowel exposed after incising parietal vaginal tunic. *Source:* Image courtesy of Richard Hopper.

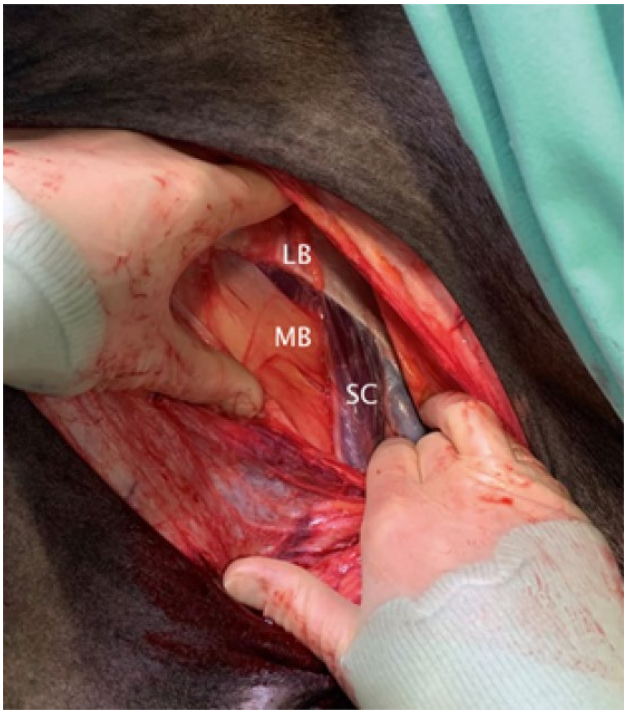


Figure 18.21 Exposure of inguinal ring. MB, Medial border of inguinal ring; LB, lateral border of inguinal ring; SC, spermatic cord. *Source:* Image courtesy of Darcie Sidelinger and Heath King.

abdominal oblique muscle posteriorly to support and protect the spermatic cord (Figures 18.21 and 18.22). An atraumatic "hernia" needle loaded with #4 non-absorbable suture is used to take the first bite through the median border of the inguinal ring near the caudal apex of the ring. The suture is passed under the spermatic cord and the second bite is made through the internal abdominal oblique muscle. The suture is then passed back beneath the spermatic cord and through the border of the inguinal ring near the first bite. This procedure is repeated on the opposite side to the spermatic cord, with the initial bite going through the lateral border of the inguinal ring to engage the internal abdominal oblique muscle 3 cm

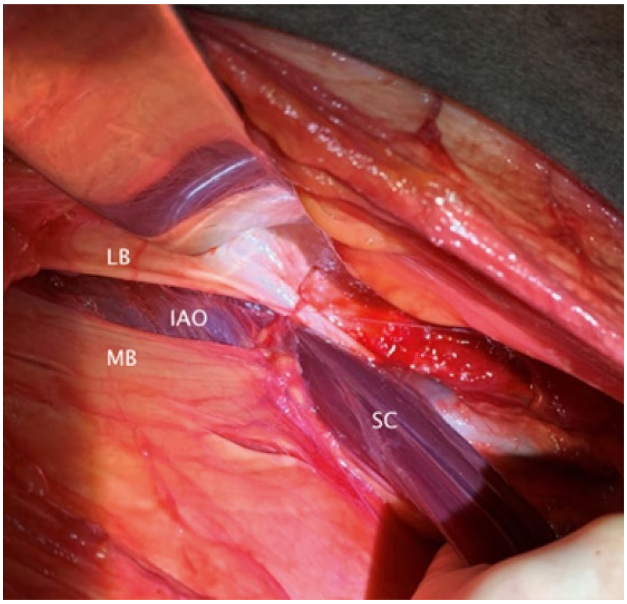


Figure 18.22 Caudal retraction of internal abdominal oblique (IAO) muscle. MB, Medial border of inguinal ring; LB, lateral border of inguinal ring; SC, spermatic cord. *Source:* Image courtesy of Darcie Sidelinger and Heath King.

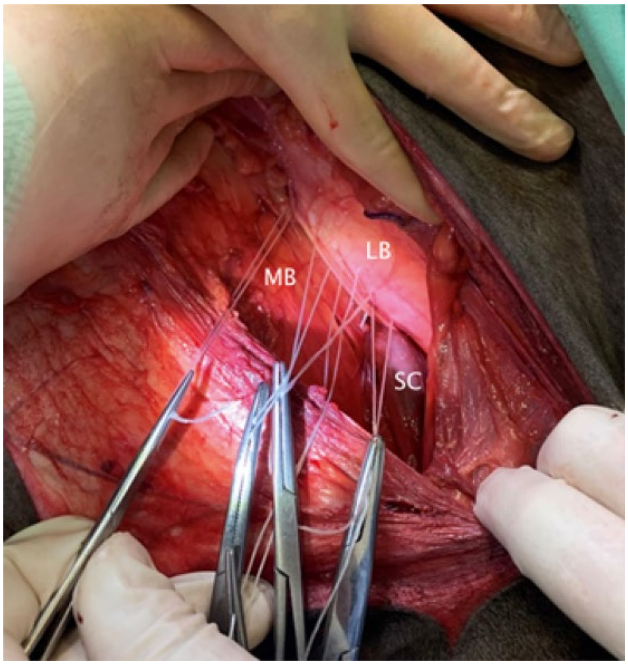


Figure 18.23 Sutures preplaced through medial and lateral borders of inguinal ring. MB, Medial border of inguinal ring; LB, lateral border of inguinal ring; SC, spermatic cord. *Source:* Image courtesy of Darcie Sidelinger and Heath King.



Figure 18.24 Completed closure of inguinal ring. *Source:* Image courtesy of Darcie Sidelinger Heath King.

from the first site. Both sutures are then tightened and tied simultaneously, with care taken to not constrict the spermatic cord. An overlapping suture pattern with non-absorbable suture is used to close the external boarders to the inguinal ring (Figures 18.23 and 18.24). Enough room should be left adjacent to the spermatic cord for two fingers to pass through the inguinal ring. The parietal vaginal tunic is closed with a simple continuous pattern of absorbable suture, and the subcutaneous tissues of the inguinal area are closed to eliminate dead space. The skin is then apposed with a continuous interlocking pattern of non-absorbable suture [17, 18].

Closure of the inguinal ring following unilateral castration is less complicated. Following ligation and transection of the spermatic cord, the lateral borders of the inguinal ring are closed with a series of preplaced simple interrupted or cruciate sutures. Large non-absorbable suture can be used or the author has also utilized #4 braided polyglactin for closure. Regardless of the approach chosen, broad-spectrum antibiotics are administered on the day of surgery and continued for five days. Non-steroidal anti-inflammatory drugs are also useful to control postoperative pain and inflammation. Recovery in a small lot versus a stall provides exercise, which helps to reduce postoperative swelling and edema.

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19

Restorative Surgery of the Prepuce and Penis

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Introduction

Bulls commonly sustain injuries to the reproductive tract during natural breeding activity. Through close observation of their bull battery during the breeding season, cattle owners or managers can identify many reproductive tract maladies. Close observation of a cowherd during the breeding season can potentially reveal cows that are returning to estrus, a bull standing off to himself, or a swelling of and trauma to the external genitalia of a bull. All of these represent issues for further investigation or veterinary intervention, which in turn should lead to the identification of conditions such as penile hematoma, preputial prolapse, phimosis, paraphimosis, or an injury that has led to a denervation, vascular shunt, or deviation of the penis. Through the utilization of a bull breeding soundness examination (BBSE) prior to each breeding season, when properly performed as directed by the standards of the Society for Theriogenology, other problems such as fibropapillomas, hair rings, and minor preputial injuries can also be identified [1].

As all of the conditions that will be described in this chapter either hamper or potentially end the functional use of a bull, it is incumbent on our part to first correctly diagnose and then provide a reasonable prognosis for successful resolution. The prognosis obviously takes into consideration the severity of the injury or condition, but also the temperament of the bull, your facilities, and economics. The economic factors that must be considered include the replacement cost of the bull, his cull value, treatment costs, and service time lost for treatment and recovery.

Fibropapilloma

Fibropapilloma or warts are a common finding in young cattle under the age of 24 months. Caused by bovine papilloma virus, the virus is believed to enter the penile skin through wounds or abrasions sustained during homosexual activity among young bulls. The virus causes neoplastic growth of fibroblasts that is not locally invasive or metastatic. Often several bulls in a group will develop penile fibropapillomas and affected bulls frequently may not have obvious lesions on other parts of the body. Other neoplastic growth on the bovine penis is extremely rare [2]. Group housing of young bulls along with a predisposition for homosexual behavior both increase the incidence and add to the possibility that the penis will be among the affected locations. Penile warts (Figure 19.1) are usually identified at the time of a BBSE [3] or by an owner that notices a young bull that is reluctant or unable to breed. Bulls may present with either a phimosis or paraphimosis.

Fibropapillomas may be managed by careful surgical removal with the bull restrained on a tilt table or in a squeeze chute [4], whether it be by excision, cryotherapy, or use of a laser, alone or in conjunction with immunization. Some level of prevention can be accomplished with the use of a commercial wart vaccine and it is my recommendation (Hopper) to administer wart vaccine to bulls following weaning that will be developed in large groups, specifically those to be shipped to bull test stations or to other group bull development stations where they will be housed with multiple bulls from multiple sources. When this is a continuous herd problem, utilization of an autogenous vaccine is a useful management tool [5].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Figure 19.1 Penile fibropapilloma identified during routine BBSE.

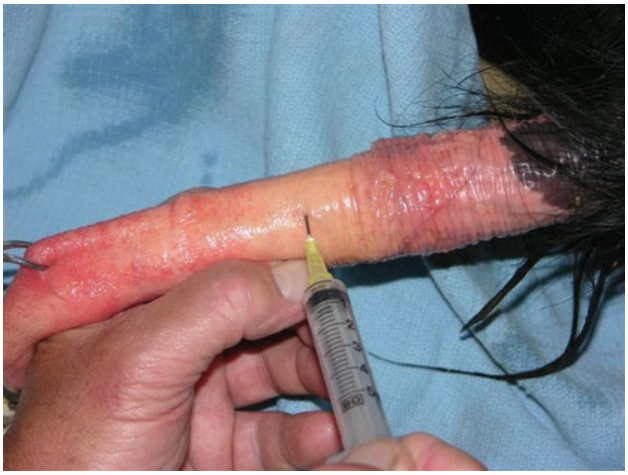


Figure 19.3 Subcutaneous injection of local anesthetic over dorsum of penis. This provides analgesia to distal penis.

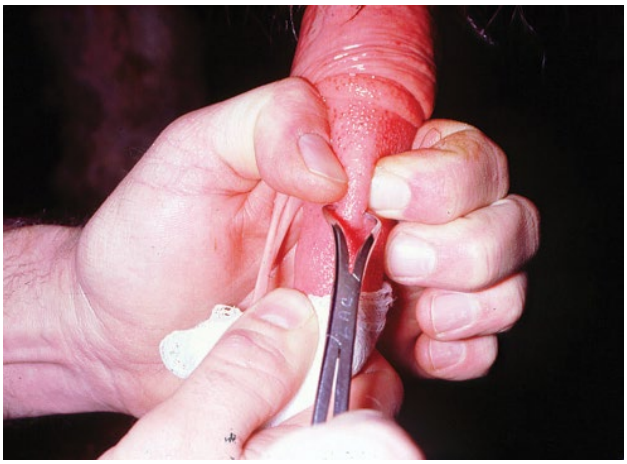


Figure 19.2 Proper placement of towel clamp under dorsal apical ligament to hold penis in extension.



Figure 19.4 Catheterization of urethra.

Surgical removal begins with manually extending the penis and placing either a towel clamp under the dorsal apical ligament (Figure 19.2) or a gauze tourniquet around the penis proximal to the growth to aid in holding the extended penis. Prepare the surgical field and infiltrate 2–4 ml of 2% lidocaine subcutaneously across the dorsum of the penis proximal to the lesion (Figure 19.3). Repeat the surgical preparation and carefully identify the urethra to avoid incising this tissue during excision of the growth. Catheterize the urethra with a 10-French male dog urinary catheter to help identify the urethra and thus avoid this structure (Figure 19.4). Then dissect the skin of the penis at the base of the lesion until the growth is completely removed. In lieu of sharp dissection with a scalpel, judicious dissection with a CO_2 laser may assist hemorrhage control. Large growths are more easily removed by gradually debulking the lesion until the entire mass is removed. Ligate any small vessels and close the skin with #0 absorbable suture. Very small growths may be excised without anesthesia or suture closure. Remove the towel clamp and return the penis to the preputial cavity. Application of topical or systemic antimicrobials is optional.

Penile fibropapilloma may recur if the bull is in an active state of the disease. Complete removal of the growth with an adjacent margin of unaffected penile surface epithelium lessens the likelihood of recurrence. In addition to the administration of a commercial or autogenous wart vaccine, anecdotal evidence suggests that the use of Immunoboost™ (Bioniche Animal Health, USA) may help prevent recurrence of penile lesions. Bulls treated for penile fibropapilloma should be examined for healing or regrowth four weeks following surgery before entering breeding service.

Hair Ring

Although far less common than warts, the presence of a hair ring also occurs due to homosexual behavior and group housing of young bulls. Accumulation of hair during riding and the continuous action of riding with extension and retraction of the penis serve to create a tight band of hair (Figure 19.5). Hair rings are typically identified during a BBSE and treatment is simply removal and application of a suitable ointment. Because the encircling ring may become sufficiently tight to cause pressure necrosis with damage to the urethra, careful examination to determine the depth of the lesion is warranted [6]. Small urethral fistulae will typically heal on their own, but those that are larger and more proximally located must be closed (Figure 19.6). In severe cases the constricting ring may cause avascular necrosis to the extent that damage to the dorsal nerves of the penis or sloughing of the entire glans penis can occur.

Persistent Frenulum: Delayed Preputial–Penile Separation

The penis of the bull calf cannot be extended prior to puberty due to the interdigitating attachment of the skin of the penis and prepuce and the lack of a sigmoid flexure. During puberty, androgen production shifts from androstenedione to testosterone, and the attachment of the penis and prepuce begins and should be complete between 8 and 11 months of age. Occasionally, young bulls between the age of 12 and 14 months will present with incomplete separation [4, 7]. In these bulls separation can be completed by pulling the prepuce back from the free portion of the penis. These tissues should separate easily and hemorrhage is seldom a problem.

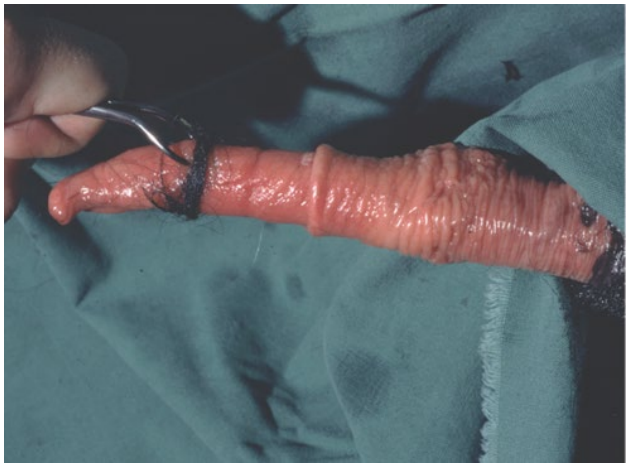


Figure 19.5 Penile hair ring.



Figure 19.6 Urethral fistula that is of a size and location to require repair.

The penile frenulum is a thin band of connective tissue on the ventral midline of the free portion of the penis, which adjoins the prepuce. Normally the frenulum ruptures during penile separation from the prepuce. When the frenulum does not rupture, the penis extends but the frenulum causes ventral bending of the distal penis during extension (Figures 19.7–19.9). Surgical repair is relatively simple. I recommend ligating each end of the frenulum and transecting the tissue to reduce the possibility of hemorrhage, although not all practitioners adhere to this practice (Figures 19.10–19.12). The owner should be advised that this condition is considered to be heritable and retaining the bull's sons as sires is not recommended.

Preputial Injury

Bulls most commonly sustain injuries to the prepuce during breeding. The extent of disruption of the surface epithelium and peri-penile elastic tissue determines the prognosis and therapeutic approach for returning the bull to breeding soundness. The veterinarian should understand the etiology of preputial injury and the therapeutic options for the animal [4, 6].

Primary preputial prolapse in the bull is usually a sequela to breeding injury, frostbite, or balanoposthitis caused by herpesvirus infection (infectious bovine rhinotracheitis/infectious



Figure 19.7 Persistent frenulum preventing complete straightening of erect penis.



Figure 19.8 Persistent frenulum with two branches attached from prepuce to penis.



Figure 19.9 Very short persistent frenulum preventing separation of penis and prepuce.

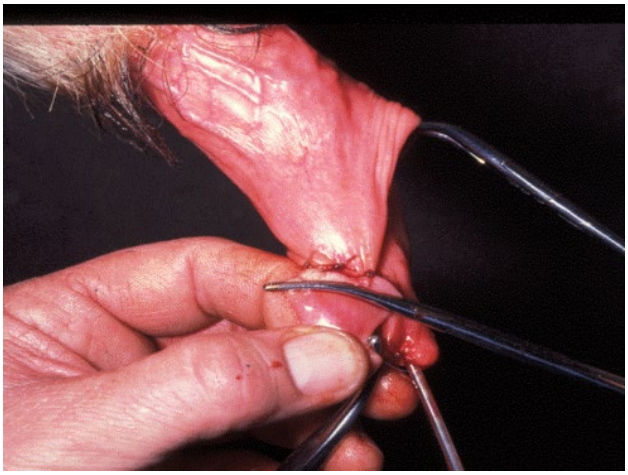


Figure 19.10 Transfixation ligatures on each end of persistent frenulum.



Figure 19.11 Excision of frenulum adjacent to transfixation ligatures.



Figure 19.12 Completed excision of frenulum.

pustular vulvovaginitis, or IBR-IPV). Secondary preputial prolapse is often seen with penile hematoma or urethral rupture. Bulls suffering preputial frostbite may undergo considerable necrosis of the preputial epithelium and heal with mild to severe preputial stenosis (Figure 19.13). IBR-IPV most commonly affects young bulls and the prepuce may be extremely edematous, with vesicles or pustules on the preputial epithelium. These bulls usually respond well to sexual rest, application of emollient ointments, and conservative therapy, which may include hydrotherapy and support bandaging.

Laceration of the prepuce of *Bos taurus* breeds usually does not lead to preputial prolapse. The damaged prepuce is typically withdrawn into the sheath and swelling of the sheath may or may not be observable. Minor injuries are often unnoticed, heal without complication, and result in only minor superficial scarring [4, 6–9]. However, it should be noted that occasionally the resultant scar tissue will create pain during breeding [1]. In this case a scar revision procedure can be utilized. Some *B. taurus* bulls with preputial laceration develop paraphimosis, where the damaged tissues will not allow retraction of the penis into the sheath (Figure 19.14). Alternatively, some bulls that do retract the penis into the sheath develop phimosis due to stricture of the injured prepuce (Figures 19.13 and 19.15).

Laceration of the prepuce with subsequent preputial prolapse occurs more commonly in *Bos indicus* breeds due to their pendulous sheath, redundant preputial tissue, and larger preputial orifice [4, 6, 10]. Additionally, when combined with cattle that are polled, this condition is exacerbated due to the fact that polled cattle breeds typically lack the preputial retractor muscle. During breeding, the excess prepuce is forced caudally and forms a collar at the preputial orifice when intromission is achieved. This collar of prepuce becomes forcefully entrapped between the bull's abdomen and the vulva and pelvis of the cow during the ejaculatory lunge, with subsequent contusion and occasionally laceration or bursting of the skin on the longitudinal axis of the ventral aspect of the prepuce. Edema quickly develops in the traumatized skin and underlying elastic tissue, leading to prolapse of the prepuce. As the penis is withdrawn into the preputial cavity the longitudinal tear assumes a transverse orientation that effectively shortens the ventral aspect of the prepuce. As edema accumulates in the damaged tissues the prolapsed prepuce increases in size and assumes the appearance of an elephant's trunk, with the lumen of the prepuce directed caudally (Figure 19.16). The laceration is evident as a transverse wound on the caudal aspect of the prolapsed tissues (Figure 19.17), with the severity of preputial tissue



Figure 19.13 Severe preputial stenosis due to frostbite.



Figure 19.14 Paraphimosis secondary to preputial laceration.



Figure 19.15 Phimosis secondary to preputial laceration.



Figure 19.16 Preputial prolapse on presentation. Note "elephant trunk" appearance.



Figure 19.18 Fresh preputial prolapse with edema and minimal necrosis.



Figure 19.17 Moderately severe preputial prolapse with tear on ventral aspect that has assumed a transverse orientation.



Figure 19.19 Preputial prolapse with severe laceration and minimal edema.

damage a result of both severity and duration of the injury (Figures 19.18–19.20).

Preputial Prolapse

Minor trauma with prolapse (Grade 1) can be treated with various medications and wrapped. More extensive trauma with swelling and the presence of necrotic tissue (Grades 2–4) requires a more aggressive approach and, because these cases result in fibrosis of the preputial tissue, surgical correction. In any case, medical treatment should be



Figure 19.20 Preputial prolapse with laceration and severe edema.

instituted as soon as possible either as the primary end of management or as a necessary precursor for surgery.

Prior to bandaging the prepuce, the wounds should be cleaned and an attempt should be made to replace the prolapse. This can be facilitated with hydrotherapy (water hose spray, showerhead spray, or soaking) (Figure 19.21). Soaking the prolapsed prepuce in a hypertonic solution with any povidone iodine solution also serves to aid in the debridement of the tissue. I (Hopper) add both salt and sugar so that solution will be very hypertonic without being too irritating. Then, after drying, an ointment is applied. I prefer a petrolatum, tetracycline, and scarlet red oil mixture for severely traumatized, necrotic wounds. I utilize less irritating ointments or mixtures for less affected tissue or after several days of treatment when the tissue is less swollen. Options for this are commercially available udder balms, human hemorrhoid ointment, intramammary infusion ointments, or "sugardine" (Betadine™ + sugar). A 6- to 10-inch rigid plastic tube (milk line or equine nasogastric tubes can be used) is then placed within the prepuce to allow urination and the prepuce is wrapped with Elasticon™. In addition to facilitating urination, the placement of the tube helps decrease the circumferential scarring of the preputial orifice, which results in phimosis. Bandage change intervals are dictated by the extent of damage, the bull's tolerance for the bandage, and whether or not you can completely replace the prepuce within the sheath. When the prepuce cannot be replaced, compression bandaging is utilized. In this case, apply the medicated ointment of your choice and cover with orthopedic stockinette prior to wrapping (Figures 19.22–19.24). In any case when the prepuce has been replaced, the use of a purse string suture to maintain retention is strongly discouraged due to the risk of abscess and an increased likelihood of stenosis. The one acceptable application for a purse string



Figure 19.21 Soaking injured prepuce.



Figure 19.22 Orthopedic stockinette applied over prolapsed prepuce.



Figure 19.23 Latex tube inserted into preputial lumen for urine drainage.



Figure 19.24 Prepuce bandage with Elasticon and with tube held in place.

retention suture would be to facilitate the safe transport to slaughter.

Bulls with a particularly pendulous preputial prolapse may benefit from a support sling to lift the sheath and prolapsed tissues close to the body. A sling fashioned by cradling the prepuce and sheath in a large piece of burlap or other loosely woven material may be held in place with bungee cords across the bull's back (Figure 19.25). The support sling may be preferred to avoid adhesive scald from repeated bandaging in bulls with especially pendulous sheath or severe prolapse with deep laceration where prolonged treatment is necessary. Alternatively, a reusable support wrap (bull diaper) also employing bungee cords over the back can be utilized (Figure 19.26). Following resolution of swelling (infection/inflammation) a decision is then made to return the bull to use or surgically correct.



Figure 19.25 Support sling fashioned from burlap material and bungee cords.



Figure 19.26 Reusable bull diaper canvass construction with eyelets for attachment to bungee cords and laundry mesh "window" for urine egress.

Surgical Management of Preputial Injury

Avulsion of the Prepuce

Avulsion of the distal prepuce from its attachment on the dorsum of the free portion of the penis occurs most commonly in bulls that serve an artificial vagina (AV) for semen collection for artificial insemination. This injury rarely occurs during natural breeding. The prepuce adheres to the latex liner of the AV and the prepuce is torn loose from the penis when the bull makes the ejaculatory lunge. Contrary to other types of lacerations, those that occur in an AV should be sutured immediately. The wounds are quickly diagnosed and comparatively clean and excellent healing is expected with immediate primary closure of the wound. If the injury is not immediately closed by suture, surgical repair should be delayed until the wound is completely covered by healthy granulation tissue. With local anesthesia of the penis, prepare the surgical field for aseptic surgery, undermine the retracted prepuce, and slide it distally to the normal position and appose the prepuce to the free portion of the penis with absorbable suture.

Ring Amputation

Often a client will choose to cull rather than allow treatment. Because these bulls are the victims of severe price discrimination, I will offer a salvage-type procedure. A large swine rectal (prolapse) ring or short length of PVC pipe is placed within the preputial lumen dorsal to the lesion and a band is placed around the prepuce over the pipe. This will amputate the damaged prepuce, but fibrosis and stenosis will likely occur. Alternatively, holes can be drilled in the PVC pipe prior to placement and, rather than a band, heavy (6-mm) Braunamid™ is utilized in an overlapping pattern. The overlapping suture serves to both fix the tube and provide for hemostasis when the end of the prepuce is amputated [3]. This is the "ring amputation" procedure and can be performed with the bull standing, utilizing local analgesia and chute restraint [1]. This procedure usually results in some degree of stricture. It should be noted that correction with the circumcision technique remains an option for bulls in which the ring amputation results in a stricture and thus it might be considered for the client that desires a more economical option than lengthy medical treatment prior to surgical correction by circumcision (Figures 19.27 and 19.28).

Circumcision (Reefing)

The surgical technique for repair that is advocated by the authors is the circumcision or "reefing" technique. The bull



Figure 19.27 PVC pipe prepared for use in "ring technique."



Figure 19.28 Ring sutured in place, inside prepuce.

must be tabled and is often anesthetized, but this procedure can be performed with regional analgesia and heavy sedation. Prior to surgery, the bull is fasted for 36–48 hours and water withheld overnight. The bull is placed in right lateral recumbency and the hair of the sheath is clipped. The penis is extended and maintained in extension with towel forceps that engage the apical ligament. The penis and prepuce are prepped (NO alcohol) and draped. A tourniquet is applied utilizing 1-inch Penrose tubing, proximal to the area to be transected. The amount of prepuce to be resected is then determined (the remaining prepuce must be a minimum length of 1.5× the free portion). Two marker sutures are placed on what will be the adjoining edges of the suture line to ensure that tissues are returned in the proper alignment. Two circumferential incisions are made with these representing the edges of the epithelial tissue to be removed. These incisions are then joined with a longitudinal incision. The incisions are to be very superficial so that with careful, sharp dissection, underlying tissue, blood vessels, and lymphatics will be spared. The area of fibrosis should be included in the tissue removed. Following dissection and tourniquet removal (the tourniquet can be maintained safely for up to 1 hour); hemorrhage is controlled by vessel ligation and/or cautery. When hemostasis is achieved, lavage the area with a warm solution of sterile saline with 50 ml Betadine Prep Solution added per liter of saline. The edges are sutured with a simple continuous subcuticular pattern using your choice of 2–0 absorbable suture material.

Do not use a single continuous pattern, but instead end the pattern and restart in three stages to avoid a constrictive (purse-string) effect. Do not close dead space. This can then be followed with a row of staples or closed with your choice of suture pattern with 0 chromic gut. Then suture in place Penrose tubing over the end of the penis. An antibiotic ointment is applied to the wound and, placing the free end of the Penrose tubing into a 6- to 10-inch rigid tube, the penis and prepuce are carefully returned into the sheath and bandaged. The bandage can stay on as long as a week; the staples can be removed in two weeks. A support wrap (Bull diaper) or sling as previously described can be employed to protect the bandage and prevent pendulant swelling (Figures 19.29–19.39).

Amputation of the Prepuce

Amputation of the prepuce is occasionally performed on young bulls of *B. indicus* breed to prophylactically shorten an excessively long prepuce to reduce the likelihood of



Figure 19.29 Penis extended in preparation for circumcision. Note loose excessive prepuce.



Figure 19.30 Determination of length of free portion of penis.



Figure 19.31 Proximal end of free portion indicated by surgeon's left forefinger.



Figure 19.32 Length of excess prepuce measured from end of sheath (surgeon's small finger) to preputial ring (surgeon's thumb).

preputial laceration when the bulls begin breeding. The advantage of this procedure is that it is quick to perform compared with circumcision. The disadvantage is that there is more elastic tissue removed, as well as underlying vasculature and lymphatics, and likely edge-to-edge apposition of skin, creating a higher incidence of preputial stenosis compared with circumcision. The surgeon should also keep in mind that the prepuce is a tubular structure and that with wound contracture stenosis is more likely.

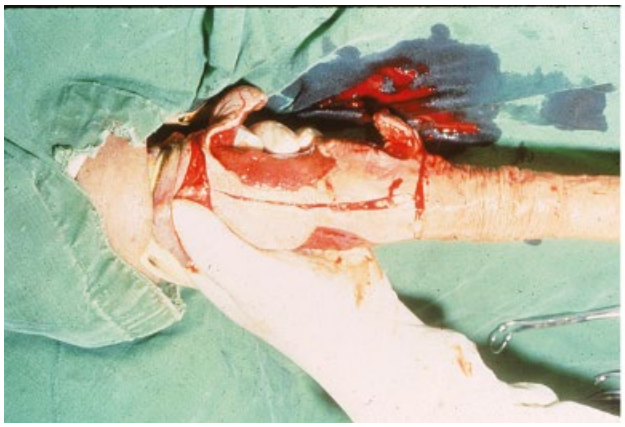
With the bull adequately restrained for aseptic surgery, infiltrate local anesthetic in the skin of the sheath just proximal to the preputial orifice. Incise the prolapsed prepuce into the preputial lumen approximately one-third of its circumference and ligate bleeders. Suture the internal and external layers of skin with #0 absorbable suture using a simple continuous pattern. Repeat the procedure for the remaining two-thirds of the circumference of the preputial prolapse. Suture a Penrose drain over the glans penis as previously described and revert the prepuce into the preputial cavity. Place an elastic bandage over the sheath as previously described and manage postoperatively as for circumcision.

Preputial Reconstruction by Scar Revision

Following healing of a preputial laceration and when the length of the healthy prepuce is insufficient to allow circumcision, the bull may be returned to breeding soundness with scar revision and preputial reconstruction. Restrain the bull and prepare the prepuce for aseptic surgery. With the penis extended, excise only the superficial transverse scar tissue (Figures 19.40–19.43). Return the edges of the preputial laceration to their original longitudinal orientation. Loosely place an absorbable bootlace suture in a longitudinal plane such that the free ends are toward the sheath (Figure 19.44). Place a 2.5-cm Penrose drain over the free potion of the penis and revert the penis into the preputial cavity (Figure 19.38). Tighten the sutures to appose the skin edges as you allow the penis to return to within the prepuce (Figure 19.45). You will restrict retraction of the penis if you tighten and tie sutures prior to this.

The purpose of this procedure is to allow the surgical wound to heal in a longitudinal plane and to maintain normal elastic tissue function. Since no elastic tissue or preputial skin is removed with this procedure, first intention healing should reduce the risk of preputial stenosis and allow subsequent penile extension. Most bulls require 60–120 days for the penis to freely extend following this procedure. Do not forcefully extend the penis during the convalescent period as the potential trauma increases the likelihood of excessive scar formation. Most bulls begin to

(a)



(b)

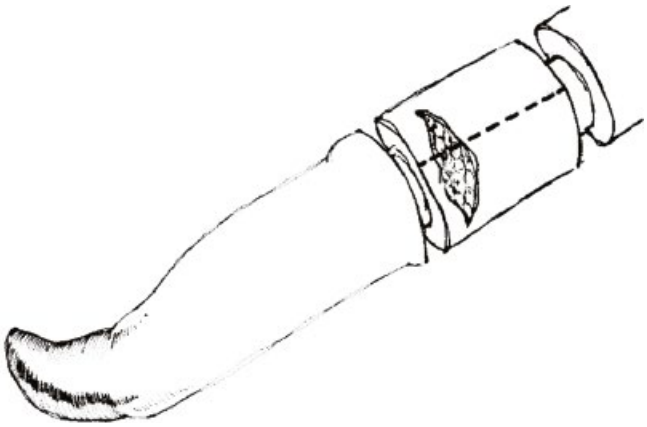


Figure 19.33 (a) Circumferential and longitudinal skin incisions in preputial epithelium. Note Proximally placed Penrose drain which serves as tourniquet. (b) Schematic of circumferential and longitudinal skin incisions for circumcision of a bull.



Figure 19.34 Dissection and removal of preputial skin and scar tissue.



Figure 19.36 Preputial epithelium suture closure.



Figure 19.35 Subcutaneous closure with continuous pattern. Note this closure performed in thirds.



Figure 19.37 Preputial epithelium closed with staples.

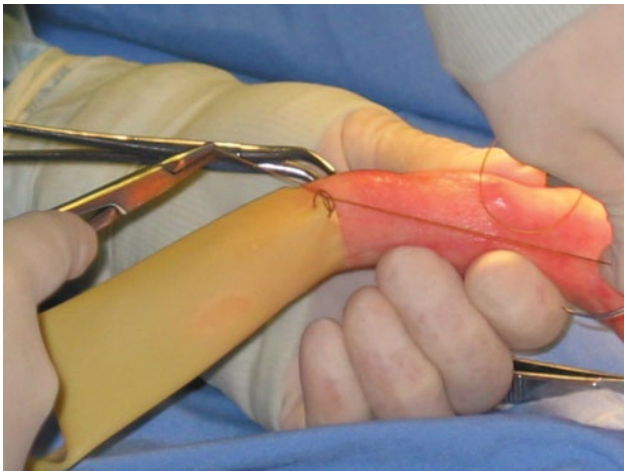


Figure 19.38 Penrose drain sutured over free portion of penis for urine drainage.



Figure 19.39 Prepuce bandaged with Penrose drain within rigid tube.



Figure 19.40 Preputial scar. This scar did not restrict extension of penis, but was painful enough to deter breeding.



Figure 19.41a Scar dissected in transverse plane.



Figure 19.41b Tissue stretched and incised area takes on oval appearance. Closure will be done in longitudinal plane with bootlace suture pattern.



Figure 19.42 Extensive fibrosis. Penis is observed following manual extension.



Figure 19.43 Scar dissected.



Figure 19.44 Closure with bootlace pattern in longitudinal orientation.

masturbate within a few weeks following surgery and will stretch contracted tissues without permanent damage.

Phimosis

Preputial injuries that occur on *B. taurus* breeds typically result in phimosis rather than prolapse and therefore represent a different sort of challenge. If the penis is forcefully extended and then not repaired and replaced within the sheath at the same time, paraphimosis may result. The prepuce can be lavaged with an antiseptic solution. Then apply an ointment of your choice and allow second intention healing to occur. If there is extensive swelling, infection, and inflammation (phelgmon), it is usually best to allow the bull one to three months to heal as much as possible on its own before attempting surgical correction. Continued

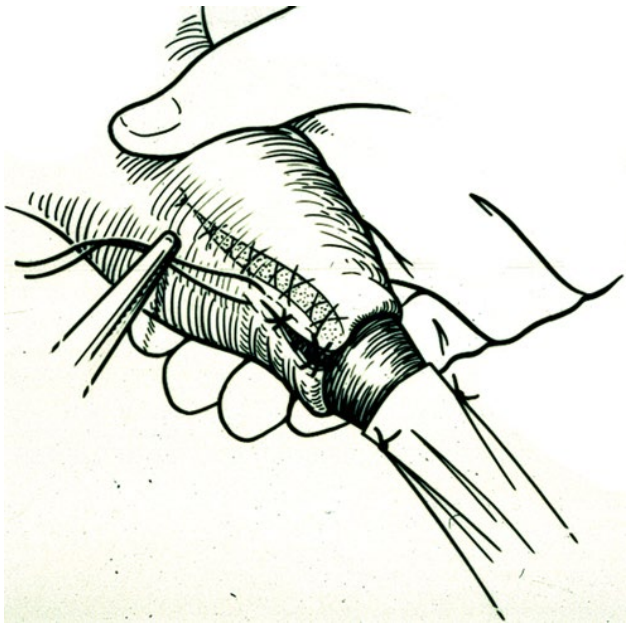


Figure 19.45 Schematic of closing incision with bootlace suture pattern when penis returned to retracted position.

treatment attempts in the face of extensive inflammation as described is virtually always unrewarding. Ascertain the extent of fibrosis and whether or not the penis can be extended (some bulls that can extend will still require surgery). In addition, it is common to encounter one or more abscesses. Scar tissue often must be removed to allow easy, painless, and full extension of the penis. Unlike *B. indicus* influenced bulls, these bulls rarely have enough preputial tissue to allow for a circumcision. A scar revision technique as previously described can usually be utilized.

Creation of Preputial Stoma

Occasionally bulls with preputial injury or frostbite develop extensive scar tissue such that there is insufficient length of healthy prepuce for scar revision and preputial reconstruction or circumcision. Creation of a stoma from the preputial cavity through the sheath may allow sufficient penile protrusion for semen collection. In my experience (Wolfe), two bulls were able to successfully complete coitus following this surgical procedure. With the bull adequately restrained and following preparation for aseptic surgery, make an elliptical incision through the skin. This incision is made on the ventral sheath sufficiently proximal to the scar tissue to penetrate the lumen of the preputial cavity (Figure 19.46). The diameter of the stoma should be sufficiently large to allow the free portion of the penis to extend, but not sufficiently large that the remaining prepuce may prolapse. Following removal of the elliptical skin, continue

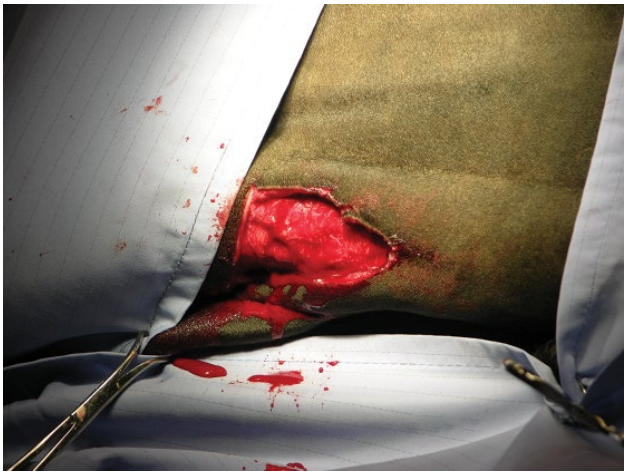


Figure 19.46 Triangular flap of skin removed from sheath (apex of triangle oriented toward scrotum).

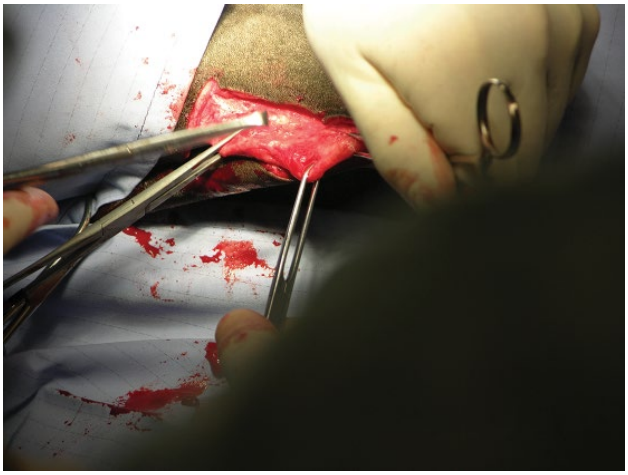


Figure 19.47 Prepuce elevated through incision in sheath.

dissection through the peri-penile elastic layers and elevate the prepuce through the opening in the sheath (Figure 19.47). Incise the prepuce (Figure 19.48) and exteriorize the free portion of the penile through the incision (Figure 19.49). Using #0 absorbable suture, appose the skin of the prepuce to the skin of the sheath with a simple interrupted pattern (Figure 19.50). Suture a 2.5-cm Penrose drain over the free portion of the penis to allow postoperative urine drainage away from the incision (Figures 19.51–19.53). Remove the Penrose drain 10 days postoperatively and allow 60 days' sexual rest prior to attempted semen collection (Figure 19.54).

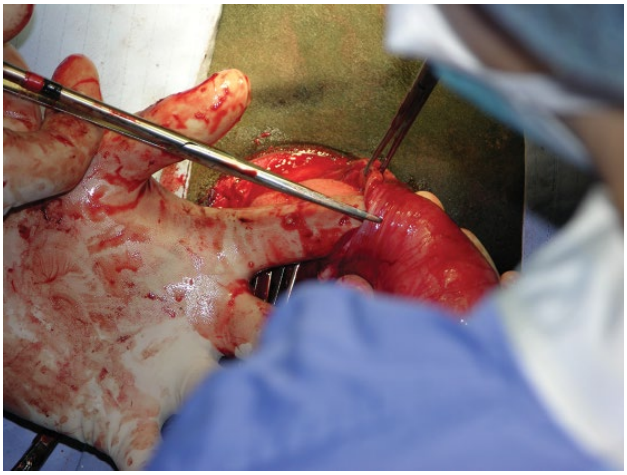


Figure 19.48 Incision into preputial cavity to create stoma.

Paraphimosis

Paraphimosis with resultant injury from penile exposure can be managed with a combination of medical and surgical therapy. Because of the inability to completely retract the penis, significant damage to the epithelial layers of the penis occurs (Figure 19.14).

Soaking the affected areas, as described previously for preputial prolapse, should occur immediately and be continued daily until there is a return to the normal appearance of the tissue. In between the therapeutic soaking the penis is covered with stockinette that has been coated in a suitable ointment (the authors' preference is one of the commercially available "bag balms"). Realize that three to four weeks of medical management is often required before the tissue is healed and surgery can be performed. Once the superficial layers are healed, a scar revision procedure as previously described to allow the penis normal retraction/extension can be performed.



Figure 19.49 Free portion of penis exteriorized through preputial stoma.



Figure 19.50 Preputial epithelium apposed to skin of sheath with interrupted absorbable sutures.



Figure 19.51 Penrose drain sutured over free portion of penis.



Figure 19.52 Penrose drain over penis through preputial stoma for urine drainage.



Figure 19.53 Postoperative image of bull with preputial stoma. (Note Penrose drain is through natural sheath opening.)



Figure 19.54 Completely healed preputial stoma ready for attempted semen collection.

Hematoma

Hematoma of the penis is probably the most common injury of the penis. This injury occurs when the penis misses the intended target, hits the cow's perineum, and bends. Although it usually occurs in young inexperienced bulls [4, 11–13] it can also occur in the older bull, which due to orthopedic injury or pain has altered his approach during mounting. From a seasonal standpoint, it usually occurs early in the breeding season when the bull is an aggressive breeder and a large number of females may be cycling, or conversely late in the breeding season when the bull may be fatigued and perhaps has lost body condition and is not as athletic or becomes awkward or clumsy during his breeding attempts. The hematoma results due to rupture of the tunica albuginea and subsequent hemorrhage from the corpus cavernosum. The initial volume of blood escaping is less than 250 ml [4], but the size of the

resultant hematoma varies as each subsequent erection results in further hemorrhage. Thus the hematoma may range in size from 15 to 30 cm. The resultant swelling occurs in the sheath over and cranial to the rudimentary teat (Figure 19.55). A prolapsed prepuce often results and this in fact may be the owner's reason for seeking help. Diagnosis may be aided with ultrasound, but, typically, a diagnosis is made from history and physical diagnosis. Do not attempt to aspirate the swelling, as inadvertent introduction of bacteria can convert a hematoma to an abscess.

Approximately 50% of bulls with rupture of the tunica albuginea return to breeding soundness without surgical repair of the injured penis. Conservative therapy consisting of absolute sexual rest for a minimum of 60 days (and avoiding extension of the penis) with systemic antibiotic therapy for the first 7–10 days is recommended. Daily hydrotherapy of the swollen sheath may stimulate circulation to the damaged tissues and assist with resorption of the edema and blood clot within the elastic tissues. If preputial prolapse is present, an emollient ointment should be applied and the protruding tissues protected with a support bandage or sling to prevent secondary trauma. The bull should have a complete BBSE prior to returning to service.

If surgery is to be performed, 5–7 days post occurrence has been shown to be the optimal timeframe [5]. Given that the owner or attendant typically may not notice this problem for 2–3 days and a 36- to 48-hour fast must occur prior to surgery, surgery must be scheduled immediately in an attempt to fit that best timeframe. Timely surgical correction avoids the clot organization and fibrosis that begins to occur 10 days post injury. Once the decision is made to attempt surgical correction, antibiotic therapy is instituted if not already begun and feed is removed. The bull is fasted for 36–48 hours and water withheld overnight. General anesthesia can be utilized or regional anesthesia with heavy sedation [14]. Either way, the bull is tabled in right lateral recumbency, with the left hind leg pulled back and up and fastened securely. The surgical area is prepped and a vertical incision 20–25 cm long is made just cranial to the rudimentary teat. Careful dissection, attention to hemostasis, and manual removal of the blood clot follow. Lavage with a warm saline + Betadine Prep Solution™ aids in the removal of additional clots and, coupled with blunt dissection, identification of the lesion (Figure 19.56). Careful blunt or manual dissection is not just preferred over scalpel or scissor dissection but mandatory, as injury to the area vasculature and nerves must be avoided. Specifically, the area near the dorsal nerve of the penis must be avoided. Since identification of this area can be confusing as penile manipulation changes the anatomical context, it is useful to identify the urethra which is on the ventral aspect of the penis and thus 180° from the dorsum.

After the tear or rent is identified, carefully lavage the area again and debride the often tattered edges of the rent. This must be minimal as excessive removal of tissue complicates closure and following healing there is the potential that the bull will have trouble extending his penis. Closure of the defect with No. 1 polyglycolic acid (PGA) in a bootlace pattern is the long-standing recommendation and justifiably so, as there is enough wound tension to make simple interrupted suture patterns problematic. The elastic layers over the penis can be closed with 3–0 chromic gut in a simple continuous pattern. The penis is then returned to its normal position within the sheath and the surgical field is irrigated with warm 3% povidone–iodine in saline. Gently remove remaining blood clots. The subcutaneous tissues are closed with 0 chromic gut and the skin can be closed with 6-mm Braunamid, typically with a Ford interlocking pattern. Antibiotics are continued for 5–7 days. The bull should have 60–90 days of sexual rest following



Figure 19.55 Hematoma location.

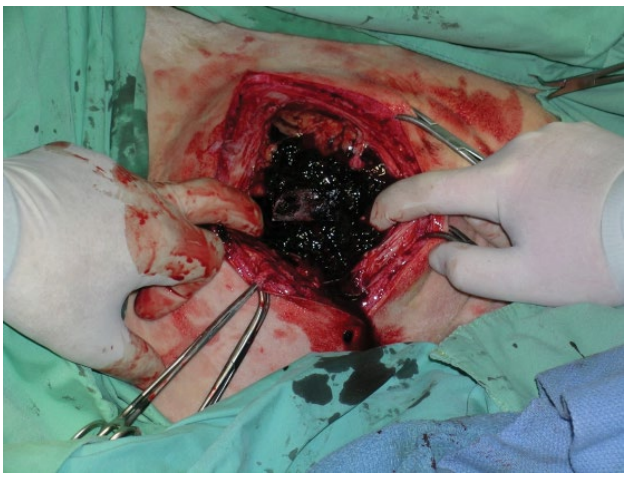


Figure 19.56 Rent in tunica albuginea.

surgery. Continue treatment of preputial prolapse as needed, but this condition usually resolves spontaneously within one week after surgery in *B. taurus* bulls. In *B. indicus* bulls the preputial prolapse may persist for longer than two weeks. In either case, it is wise to evaluate them for secondary damage to the prolapsed tissues. Seroma formation occurs often enough that it probably should not be considered a true complication. Complications include abscessation, suture dehiscence, shunts, permanent analgesia to the penis, and reoccurrence during the subsequent breeding seasons [2, 14]. Permanent analgesia may be the result of the original injury or an injury that occurs even following surgical resolution of the hematoma. In this later instance, the bull, having returned to service, sustains penile analgesia after breeding a few cows. Best evidence suggests that there are adhesions that surround portions of the nerves along the distal bend of the sigmoid flexure. These adhesions are not severe enough to restrict extension of the penis, but following repeated breeding they tear enough to damage the nerves [14].

Penile Deviations

Deviations of the penis may occasionally prevent intromission and coitus. Bovine penile deviations are classified as spiral (corkscrew), ventral, or S-shaped [4, 15–19]. Typically, bulls have had one or more successful breeding seasons prior to development of penile deviations with usually no known history of previous penile injury. Most bulls with these conditions are three or four years old and occasionally a bull over that age will develop penile deviation.

Spiral Deviation

Spiral deviation of the penis occurs more commonly than either ventral or S-shaped deviations. One potential etiology involves malfunction of the dorsal apical ligament. The apical ligament, a thick collagen band, arises from the dorsum of the tunica albuginea about 2.5 cm proximal to the distal end of the prepuce and inserts into the tunica albuginea near the distal end of the corpus cavernosum penis (CCP). The function of this structure is to help maintain the shape of the penis during erection [17]. Historically, spiral deviations were considered to result from a short or inadequate apical ligament that slipped off to the left side of the penis at peak erection, allowing the penis to spiral and therefore preventing intromission [16–18].

However, the rapidly coordinated events leading to erection and the high maximum pressures within the CCP at ejaculation suggest that affected bulls may prematurely achieve maximum erection pressures prior to intromission. This suggestion may be supported by the fact that the penis of many normal bulls likely develops a spiral orientation in a cow's vagina during ejaculation [19]. Although the definitive cause of spiral deviations remains uncertain, apical ligament malfunction and premature achievement of high CCP pressure may be involved.

Many bulls develop spiraling of the distal penis during electroejaculation and masturbation [20]. Therefore spiral deviation should only be diagnosed as a pathologic condition when it consistently develops during natural mating (Figure 19.57) [4, 17, 21].

Bulls with spiral deviation of the penis are most often three years old and have had a successful previous breeding season. Development of this condition may be insidious, occurring intermittently at first, then more consistently as breeding efforts continue. Therefore several test matings may be necessary to confirm the diagnosis. In *B. indicus* bulls the deviation may occur prior to penile extension such that the deviation remains hidden within the bull's pendulous sheath and excessive prepuce. For bulls with this sheath conformation, manual retraction of the prepuce during attempted breeding may be necessary to differentiate from erection or other causes of penile extension failure.

Ventral Penile Deviation

Ventral penile deviation occurs much less commonly than spiral deviation and the etiology is uncertain. Ventral deviations may occur as a result of altered blood flow through the ventral portion of the CCP or due to stretching or injury of the apical ligament, both of which probably result from chronic traumatic injury [1, 17, 18].



Figure 19.57 Spiral deviation.

Ventral penis deviation is obvious as long gradual curvatures of the erect penis (Figure 19.58). The curvature frequently originates proximal to the junction of the sheath and prepuce [17, 18]. These conditions become more apparent as erection pressure increases. Because ventral penile deviation can be induced during electroejaculation, observation during natural breeding is recommended, especially for less obvious cases. These conditions are only considered to be pathologic when they consistently prevent intromission.

Repair of Spiral and Ventral Penile Deviations

The prognosis for return to breeding soundness is greater following surgical repair of spiral than of ventral deviations. I recommend only attempting surgical repair of ventral deviations when the deviation is limited to the free portion of the penis. Spiral and ventral penile deviations are both repaired with a fascia lata graft. A narrow strip of fascia lata is sutured between the dorsal apical ligament and the dorsum of the tunica albuginea to serve as a fibroblast lattice to strengthen adjacent structures and stabilize the apical ligament on the dorsum of the penis [22].

Fascia Lata Graft Technique

This deviation as with those of a developmental etiology can be repaired with a fascia lata implant technique described by Walker and Young [22]. A rectangular strip of fascia is harvested from the bull, cleaned (areolar tissue removed), and placed between the apical ligament and the tunica albuginea. Alternatively, synthetic surgical mesh material can be used to substitute for the fascia implant. Utilization of the mesh has the obvious advantage of removing the time-consuming fascia harvesting step. However, problems with postoperative infection with the mesh materials of the day along with dissatisfaction with the apical ligament "strip" technique were the impetus stated by Walker for the development of this technique [22]. Thus, the fascia lata technique will be described and those that prefer to can easily modify the technique to utilize surgical mesh.

The bull is fasted for 48 hours and water withheld overnight. Depending on his nature, the bull can be sedated with 10–20 mg xylazine and 10 mg acepromazine IV. With the bull restrained and standing, the surgical site, an area on the upper left hindlimb, is prepped, since the bull will later be placed in lateral recumbency on his right side. A local block utilizing an inverted L injection pattern is administered. A 15- to 20-cm incision is then made utilizing the patella and greater trochanter as anatomical guides, with the incision being midway between. When the fascia lata is exposed, remove a rectangle-shaped section. Removing a 3-cm-wide by 15-cm-long section will provide more than enough tissue for your graft (Figure 19.59). Place the tissue in saline, maintaining sterility, and suture the edges of the fascia with a continuous pattern utilizing any No. 1 or 2 dissolvable suture. Failure to do so will result in painful muscle herniation. The skin can be closed with No. 3 Braunamid utilizing a Ford interlocking pattern. The harvested tissue is prepared by rinsing in saline and removing any attached tissue (Figure 19.60).

The bull is then prepared for the placing of this graft material. As with the other surgeries described, general anesthesia can be utilized or regional anesthesia with heavy sedation. The bull is tabled in right lateral recumbency and the preputial area is clipped and prepped. The penis is extended and the apical ligament identified and grasped



Figure 19.58 Ventral deviation.



Figure 19.59 Harvest of fascia.



Figure 19.60 Prepared fascia graft.

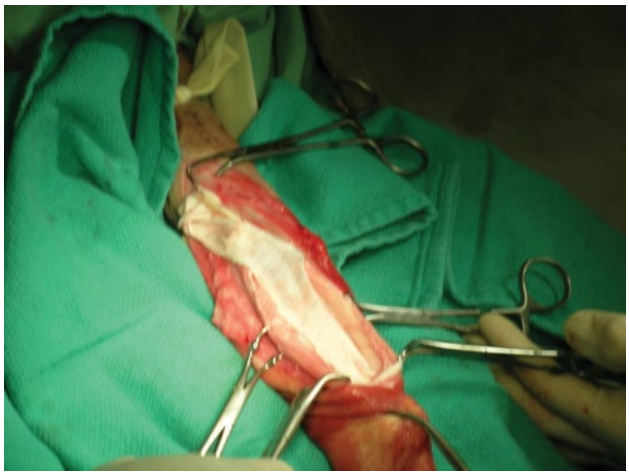


Figure 19.61 Placement of fascia graft.

with towel forceps. The penis is prepared with Betadine Surgical Scrub™, rinsed with sterile water or saline, and dried. Unlike when performing a circumcision, a tourniquet need not be utilized, as you will want to be able to easily visualize the area vasculature. An incision is made on the central dorsal aspect of the penis from a point 3 cm from the tip and extending 20 cm proximally. With careful dissection, identify the apical ligament and incise through it for its entire length. This incision will expose the tunica albuginea, and this is where the fascia or alternatively the synthetic mesh implant will be placed (Figure 19.61). The proximal aspect is placed first and sutures (2–0 chromic gut) are placed in the corners attaching the implant material to the tunic. Interrupted sutures are then placed on the lateral sides of the implant, stretching the implant so as to avoid crumpling of the tissue (Figure 19.62). Care is taken to not penetrate too deeply into the tunic and to avoid suture placement that impinges on the dorsal vasculature.

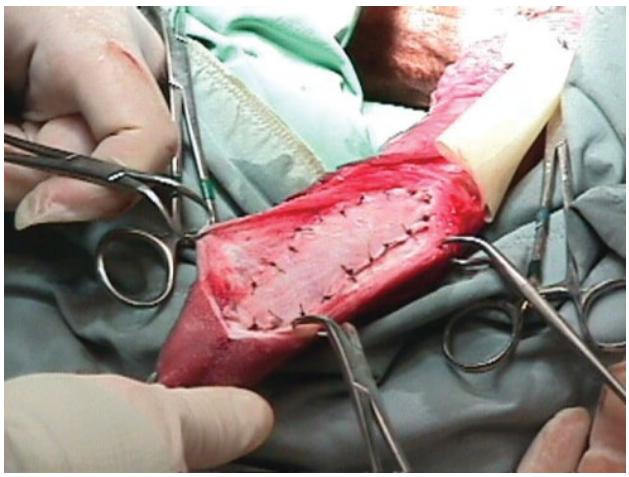


Figure 19.62 Suturing graft to tunic.

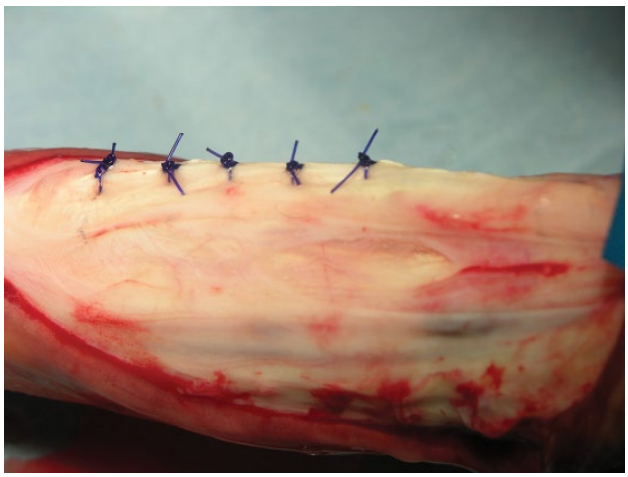


Figure 19.63 Closure of apical ligament.



Figure 19.64 Closure of penile and preputial epithelium.

The distal end of the implant is trimmed if necessary and the distal end is sutured as previously described.

The edges of the apical ligament are closed utilizing 0 chromic gut, with every other or every third suture engaging the implant (Figure 19.63). This suture will keep the apical ligament from slipping to the side, which is crucial if correcting a spiral deviation. The elastic tissues and dead space are closed with a continuous suture pattern (3–0 or 4–0 chromic gut) and the skin can be closed with 0 chromic gut or other dissolvable suture using an interrupted pattern (Figure 19.64). The incision site is gently rinsed with dilute Betadine solution, an antibiotic ointment (bovine intramammary infusion medication) is liberally applied, and the penis is returned to the prepuce. Systemic antibiotics are administered for 4–5 days and the penis can be manually extended for examination in 7–10 days. Three months of sexual rest is recommended.

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20

Management of Urolithiasis

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Introduction

Urolithiasis is an economically significant disease of ruminants. In cattle, fatal urolithiasis is reported anywhere from 3 to 10% [1, 2]. Treatment and prevention of urolithiasis is a continual challenge for bovine practitioners and producers. The morbidity and mortality is devastating for not only steers in feedlots, but also breeding soundness for bulls.

Urolithiasis is a multifactorial disease involving diet, urine pH, and body water balance. Contributing factors include ruminant anatomy, factors favoring nidus development, factors precipitating solutes, and factors favoring cementing of stones. Uroliths occur when minerals precipitate from the urine and bind to an organic matrix or nidus [3]. The organic matrix is a mixture of urinary peptides, proteins, and mucopolysaccharides [3]. The proteinaceous aspect of the matrix can consist of desquamated epithelial cells, casts, or other urinary tract debris for infection or inflammation; however, this is uncommon in ruminants [3].

In cattle, urolithiasis typically falls into two categories: animals consuming high concentrate rations and pastured animals consuming grasses high in silica [4]. High concentrate rations usually fed with feedlot and show animals contain a low calcium to phosphorus ratio. High phosphorus rations typically create phosphate calculi (calcium, ammonium, and magnesium), with struvite being the most common [4, 5]. Ruminants afflicted with phosphate urolithiasis typically have multiple offending calculi that are smooth, soft, and sandy in appearance. These are the uroliths typically associated with calculi on the preputial hairs. Cattle grazing pastures in the western USA high in silica are most at risk of developing silicate uroliths. Silicate uroliths can even occur after removal of the offending forage [4]. Oat grain and oat straw are also high in silica and can be the etiology of silica urolith in the absence of grazing a pasture high in silica [4]. Cattle afflicted with silica urolithiasis typically obstruct with a single urolith that is rough, hard, and white measuring approximately 4–7 mm in diameter. One retrospective study reported 6.6% of cases afflicted with two distinct calculi [5]. Carbonate and oxalate uroliths are uncommonly encountered in cattle [4]. Carbonate uroliths are associated with consumption of legumes and oxalate uroliths are associated with consumption of oxalate-containing plants such as clover, halogeton, greasewood, soursob, and sorrel [6].

Uroliths tend to form in alkaline urine in ruminants with normal pH ranging from 7 to 9.5. Increasing urine pH results in increasing likelihood that urinary colloids will precipitate [7]. Uroliths that precipitate in alkaline urine include struvite, calcium phosphate, and calcium carbonate [4]. Urine pH has no effect on the calculogenesis of silica uroliths [3, 4].

Inadequate body water balance is a significant risk factor in the occurrence of urolithiasis. Dehydration results in increased solute concentration in the urine, providing more scaffold and calculogenesis opportunities. Seasonality can occur, with an increase of cases seen when decreased water consumption occurs. During the summer, palatable water sources and drought can result in decreased water intake. During times of extreme cold, voluntary decreased intake may occur. Herd outbreaks of urolithiasis can even occur if available palatable water sources are extreme.

The narrow, long, and tortuous male ruminant urethral anatomy contributes to the occurrence of urinary obstruction in cattle. The most common site for urethral obstruction in bulls and steers is the distal sigmoid flexure. Occurrence at the ischial arch is less commonly reported [8]. While urolithiasis is seen in castrated and intact males, obstruction tends to be most common in steers due to the narrower urethral diameter from the absence of testosterone. Early castration

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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has been shown to result in decreased urethral diameter and increased incidence of urolithiasis [9, 10]. Steers greater than eight months of age are the most commonly presented for disease [9]. Because of the effect of castration on urethral diameter, some authors suggest delaying castration until six months of age [1]; however, ethical considerations and castration complications at this weight should be considered before implementation of this recommendation.

Other commonly cited implications for urolith formation include hypovitaminosis A, hypervitaminosis D, and estrogenic intake [1, 4, 8]. However, true evidentiary support in the literature is lacking. Hypovitaminosis A occurs when cattle are fed poor quality hay and silage stored for prolonged periods. Hypervitaminosis D is commonly a result of feeding error during ration mixing. Diethylstilbestrol implantation and estrogen intake are hypothesized to result in hypertrophic effect on seminal vesicles, urethras, and bulbourethral glands, thus increasing the risk for urolithiasis [1, 8, 10].

Clinical Signs

Clinical signs associated with urolithiasis vary based on the location and duration of the obstruction. Common clinical signs include listlessness, anorexia, colic, tail switching, posturing, vocalization (more common in goats), and dehydration. Less common signs include rectal prolapse associated with tenesmus. For urolithiasis associated with phosphate calculi, stones can be appreciated on the preputial hairs (Figure 20.1). For cases with a single obstructive urolith, careful palpation of the urethra along the length of the penis might assist with location of the urolith.



Figure 20.1 Urethral calculi visible on preputial hairs.

Acute urethral obstruction is commonly associated with acute colic symptoms such as bruxism, repeated stretching, treading of feet, and kicking of the abdomen in bulls and steers. In addition to the previously mentioned clinical signs, palpation of the preputial orifice and preputial hairs is dry, indicating no urine passage. Upon rectal palpation, affected individuals will have an enlarged urinary bladder and palpable urethral pulsations. Individuals with a partial urethral obstruction show signs of discomfort as well as dribbling of urine, stranguria, and hematuria. If a complete obstruction is left untreated for 48 hours or longer, a urethral rupture or bladder rupture will occur and signs of colic typically cease. If a urethral rupture occurs, subcutaneous edema develops along the ventral abdomen that can extend from the scrotum to the sternum, but especially in the preputial region (Figure 20.2). Severe necrosis and sloughing of these tissues can occur if left untreated. With bladder rupture, bilaterally symmetric ventral abdominal distention will occur ("waterbelly"). With bladder rupture, a palpable fluid wave will also be present. Upon rectal palpation, free abdominal fluid will be appreciable and a small partially filled urinary bladder. Bulls with uroabdomen may also develop a hydrocele. Owners typically report the colic associated with a complete urethral obstruction, then improvement for 24–48 hours, after which they notice the abdominal distention and worsening depression again. Dehydration is also severe (≥10%) with bladder rupture, due to osmotic pull of fluid from the vascular space into the abdomen. Individuals afflicted with ureteroliths and nephroliths will experience similar clinical signs as those listed above. A ureterolith and hydroureter may be palpable per



Figure 20.2 Subcutaneous peripenile swelling typically extends from the base of the scrotum cranially and involves the entire ventrum.

rectum, but often definitive diagnosis of ureteroliths and nephroliths does not occur antemortem.

Diagnosis

Urolithiasis can typically be diagnosed utilizing historical data and clinical signs. Additionally, rectal palpation and careful palpation of the penis can typically locate the site of obstruction and the presence or absence of a urethral or bladder rupture. For feedlot steers or economically constrained cases, treatment is implemented based on physical examination. However, for valuable individuals a complete diagnostic picture includes a complete blood count (CBC), biochemistry profile, and ultrasound.

The abnormalities observed with a CBC will typically coincide with the severity and duration of disease. Typical findings include a neutrophilia and possible hemoconcentration depending on the degree of dehydration. Cases with urethral rupture may have increased fibrinogen with excessive subcutaneous tissue necrosis. Common biochemical abnormalities include azotemia, hyponatremia, and hypochloremia. In ruminants, potassium can be normal or elevated in individuals with obstructive urolithiasis. When hyperkalemia is present, the degree of elevation is typically mild. One study experimentally induced uroabdomen and demonstrated increased salivary potassium excretion, possibly explaining the less severe hyperkalemia observed in ruminants with urolithiasis [11]. In small ruminants, Ewoldt et al. found individuals with no free abdominal fluid and a serum potassium less than 5.2 meq/l more likely to survive [12]. Individuals with urolithiasis may have a metabolic alkalosis, acidosis, or normal blood pH. When acidosis occurs, individuals are often severely dehydrated and suffering from lactic acidosis [13]. With prolonged obstructions, hyperphosphatemia and hypomagnesemia can occur and concerns of secondary renal failure should be considered. One study stated the best prognostic indicator was excessive serum phosphate being associated with increased likelihood of death [14]. In absence of hydronephrosis, 48 hours of fluid diuresis should be administered before diagnosing an individual in secondary renal failure. In individuals with uroabdomen, the degree of azotemia, hyponatremia, hypochloremia, and hyperkalemia can be severe due to the osmotic gradient across the abdominal wall pulling sodium and chloride into the abdomen, potassium and blood urea nitrogen intravascularly, with creatinine staying mostly intra-abdominally, since it diffuses at a slower rate being a larger molecule.

If available, ultrasound can be used to assist with assessment of the urinary tract. Ultrasonography can be used transcutaneously or transrectally to assess the integrity and location of uroliths. Additionally, ultrasonography can assist with diagnosing the presence of cystoliths, ureteroliths, or nephroliths. Cystoliths usually have a sandy debris appearance that is hyperechoic with a rough, irregular surface that casts acoustic shadows [15]. Ureteroliths and nephroliths are often difficult to diagnose antemortem, but are hyperechoic half-moon-shaped or round calculi that cast acoustic shadows through deeper tissues [15]. Ureteroliths may also be diagnosed via rectal examination if hydroureter is associated with the condition. Prior to surgical intervention, presence of hydronephrosis and/or hydroureter should be investigated. The right kidney extends from the 13th rib to the third lumbar vertebrae with the cranial pole in the renal impression of the liver in the 12tth intercostal space [15]. The left kidney can be imaged from the right paralumbar fossa caudal and is found slightly ventral to the right kidney from the 2nd to 5th lumbar vertebrae [15]. The left kidney can also be evaluated with transrectal ultrasound. If severe hydronephrosis is present, humane euthanasia is recommended (Figure 20.3). Presence of peripenile subcutaneous edema is suggestive of a urethral rupture and poor prognosis for returning to breeding soundness. Significant diffuse hypoechoic free fluid in the abdominal area is generally suggestive of uroabdomen. However, definitive diagnosis of uroabdomen is done by performing an abdominocentesis in either the flank fold of the abdomen or at the location of the fluid found ultrasonographically with an 18-gauge, 3.8-cm (1.5-inch) needle after surgical prep. An abdominal fluid creatinine to serum creatinine ratio of 2:1 or greater confirms the diagnosis of uroabdomen [11, 16].

A baseline urinalysis can be performed. Common abnormalities include hematuria, proteinuria, and alkalinuria. Diagnosis of bacteriuria is beneficial to ensure a primary urinary tract infection is not the etiology of the urolithiasis.

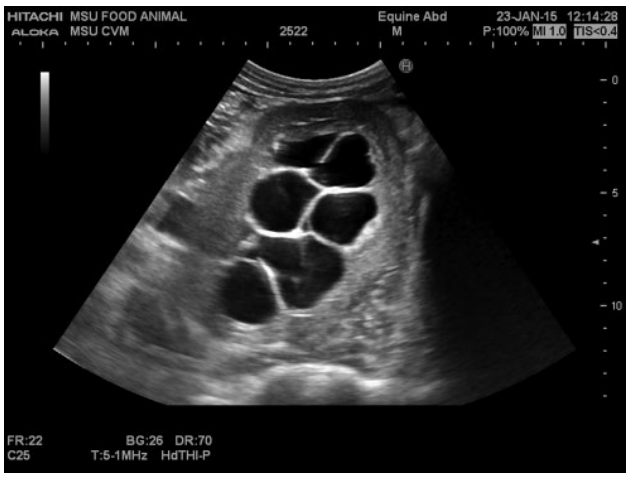


Figure 20.3 Severe hydronephrosis in a mature bull.

Baseline pH knowledge is important if urinary acidification is implemented. Ideally, if uroliths are obtained during surgery, the uroliths should analyzed to determine type. This information will help determine the best prevention strategy for the individual and herd. If urolith analysis is not feasible or delayed, a urolith can be placed in an acidifying solution to determine if urinary acidification would be a useful adjunct therapy. If available, urethroscopy can be utilized to assess location of the obstruction, extent of urothelial damage, and presence of strictures, or provide possible avenues for utilization of a laser or basket retrieval method.

Treatment

Immediate Care

Prior to initiating treatment for urolithiasis, the intended use of the animal, economics, site of obstruction, and integrity of the urinary tract should be considered. For acute cases, medical management can be attempted or immediate slaughter if uremia is not present. Most cases of urolithiasis will require surgical intervention. For breeding bulls, maintaining a patent urethra is paramount for successful outcome and breeding career. Surgical procedures utilized in breeding individuals include tube cystostomy, cystotomy, ischial urethrostomy, and urethrotomy. Surgical procedures utilized in salvage bulls and steers include ischial urethrostomy, perineal urethrostomy, and urethrotomy.

Before sedation or anesthesia, any severe electrolyte derangement should be corrected. For severe hyperkalemia, a bolus of 50% dextrose can be administered for an endogenous release of insulin to drive potassium intracellularly. Exogenous insulin can also be administered; however, this should be used with caution to avoid iatrogenic hypoglycemia. A bolus of hypertonic bicarbonate (8.4%) can also be administered to drive potassium intracellularly in individuals without metabolic alkalosis. For individuals with bradycardia caused by severe hyperkalemia, calcium borogluconate and atropine can be administered.

Saline (0.9%) is a good empirical choice for fluid therapy in treatment of urolithiasis. Saline will help correct the hyponatremia and hypochloremia often present in individuals with urolithiasis and will not exacerbate the hyperkalemia. However, if metabolic acidosis is present, saline should be avoided. For individuals greater than 8% dehydrated, intravenous fluid therapy is ideal. However, in field circumstances or economic constraints, hypertonic saline and oral fluids provide a reasonable alternative. In severe hypovolemia, fluid therapy should be instituted prior to sedation or tranquilization. Due to the diuretic effect, alpha 2-adrenergic agonists should never be used for tranquilization in cattle with urethral obstruction unless administered for sedation immediately prior to surgical relief.

Urethral rupture is diagnosed during physical examination. If present, this often will dictate the outcome and treatment plan for the individual. Generally, once a urethra has reached the point of rupture, severe urethral necrosis has occurred and attempting primary closure is futile. Typically, a perineal urethrostomy and salvage is the treatment recommendation with urethral rupture due to the high occurrence of urethral stricture at the site of rupture. If breeding soundness is desired, a urinary diversion technique such as a tube cystostomy or ischial urethrostomy should be performed to allow the site of urethral rupture to heal by second intention. However, prognosis for natural breeding soundness is poor due to the likelihood of peripenile adhesions from subcutaneous urine accumulation, as well as the possibility of urethral stricture and fistula formation. In cases with severe subcutaneous urine accumulation, skin incisions should be performed as needed to facilitate drainage to help reduce the degree of tissue necrosis, infection, and abscessation that occurs caused by the caustic nature of urine.

For cases with bladder rupture and uroabdomen, stabilization is required prior to pursuing surgical intervention. Ideally, intravenous fluids should be administered to correct severe dehydration and electrolyte disturbances prior to general anesthesia. For feedlot steers, hypertonic saline and oral fluids are a more economical choice. Drainage of urine from the abdomen should be performed with all cases of uroabdomen, but it is especially important to perform prior to placing any individual in dorsal recumbency. The drainage site should be surgically prepped and infiltrated with 2% lidocaine. A rumen trocar and cannula or thoracic catheter can be used to tap the abdominal cavity just medial to the caudal superficial epigastric vein [4]. The fluid should be drained slowly over 30 minutes or more preferably with intravenous fluid support to prevent circulatory shock. Rapid removal of uroabdomen at surgery should also be avoided because hypotensive crisis and death can occur. Ideally, the abdomen should be lavaged during surgery. However, cattle with uroabdomens do not develop peritonitis in experimentally created uroadodomen, so removal of urine is typically sufficient [11]. For valuable breeding animals, a cystorrhaphy is ideally performed via a standing laparotomy or ventral paramedian incision. However, access to the bladder can be difficult with a standing approach and often uroabdomen is the result of multiple small holes secondary to chronic overdistension and necrosis rather than a single rent. Often a ruptured bladder will heal without primary surgical repair. As long as the urinary bladder remains decompressed via a

tube cystotomy, ischial urethrostomy, or indwelling urinary catheter, the rent in the bladder will form a fibrin seal and heal. A ruptured bladder can also heal with a perineal urethrostomy if urethral patency is established. Ventral tears are less likely to heal without primary surgical repair. Spontaneous healing of the bladder occurs in approximately 50% of animals following urethrotomy and drainage of the abdomen [17]. Cases of experimentally induced uroabdomen survived an average of 14 days until death [11]. The survival rate in steers with urinary bladder rupture is reportedly 90% in animals treated with tube cystostomy [18] compared with 55% [19] and 86% [20] in animals treated with cystorrhaphy, with or without urethrotomy and transurethral catheterization, respectively.

Unless an individual is to be immediately slaughtered, antibiotic therapy should be administered in preparation for any surgical intervention. Additionally, antimicrobials should be considered in cases managed with retrograde catheterization or where a urinary tract infection is suspected. Generally, beta-lactams are appropriate due to their renal excretion. Antibiotics should be administered five to seven days postoperatively. For cases treated with an indwelling urinary tube or catheter, antibiotics should be administered for the duration of catheter or tube placement and continued for three to five days after removal.

Pain management should be administered to individuals treated medically and surgically. For postoperative pain, inflammation, and urethral swelling, non-steroidal anti-inflammatory drugs (NSAIDs) are recommended. Flunixin meglumine (1.1 mg/kg intravenous [IV]) is administered perioperatively for pain and inflammation. Caution should be used when administering flunixin meglumine to extremely dehydrated patients if immediate rehydration is not performed. Meloxicam (0.5–1 mg/kg per os [PO]) can be administered as needed postoperatively for pain. In cases where NSAIDs are insufficient for pain management, butorphanol (0.025–0.05 mg/kg intramuscularly [IM] or subcutaneously [(SC]) can be administered every six to eight hours in conjunction with NSAID therapy. Typically, the addition of opioids is only required for the first 24–48 hours postoperatively.

For cases with phosphatic calculi, urinary acidification is usually essential for treatment success. Ammonium chloride is recommended at 200 mg/kg orally every 24 hours for urine acidification. Due to the risk of causing metabolic acidosis, it is acceptable to start at 50 mg/kg orally every 24 hours and increase by 50 mg/kg until desired urinary pH is achieved. Phosphatic calculi will dissolve at a urinary pH range of 6–6.5, so the dose of ammonium chloride should be increased until this is achieved. Daily urinary pH should be monitored until a pH of 6–6.5 is reached. Feeding a dietary cation–anion difference (DCAD) ration is also an option to cause urinary acidification, but this is often difficult to implement for a single individual. In our clinic, we will often feed Soychlor®. Soychlor is utilized for DCAD rations in dairy cattle to aid in the prevention of hypocalcemia. For the cases in our clinics, we often feed 0.1–0.2 kg (0.25–0.5 lb) mixed with grain to create acidified urine. Soychlor eliminates the need for forced PO administration of ammonium chloride utilizing a balling gun to a patient and is easier than DCAD ration formulation. Urine acidification only needs to be performed until the clinician feels the stones dissolve due to the ruminant's ability for renal compensation and realkalization of urine. Further, long-term use of ammonium chloride can result in osteopenia.

Medical Management

Medical management is often unrewarding in the treatment of urolithiasis unless instituted early in the disease course. If the obstruction is recognized early, conservative therapy can be attempted with spasmolytic drugs and tranquilizers [2, 21, 22]. Phenothiazine tranquilizers are typically utilized which decrease urethral spasm and induce relaxation of the retractor penis muscles and straightening of the sigmoid flexure [2, 22]. In early cases, straightening of the sigmoid flexure and decreased urethral spasms may help urethral calculi to pass. However, if unsuccessful, retrograde urethral catheterization with hydropulsion can be attempted.

Retrograde catheterization is most easily performed in mature bulls versus castrated or prepubertal males due to the presence of persistent frenulum. Once exteriorization of the glans penis is accomplished, retrograde catheterization can be attempted with 8- to 10-French polypropylene tubing. Infusion of lidocaine may assist with urethral spasm prior to attempting hydropulsion with saline. If significant resistance occurs, further attempts should be abandoned to prevent further urothelial trauma, and surgical intervention is necessary. Catheterization and hydropulsion are typically unsuccessful in cattle due to the presence of the urethral diverticulum and the chronicity in which most individuals present.

Surgical Management

Tube Cystostomy

A tube cystostomy is associated with the best long-term prognosis in small ruminants for survival and breeding [12, 23–25]. This method provides urinary diversion, allowing the relief of urethral spasm and swelling and allows the urothelium time to heal. A tube cystotomy can be performed in dorsal recumbency via a paramedian incision or standing (Figure 20.4) via a left flank laparotomy (Figure 20.5) [26].

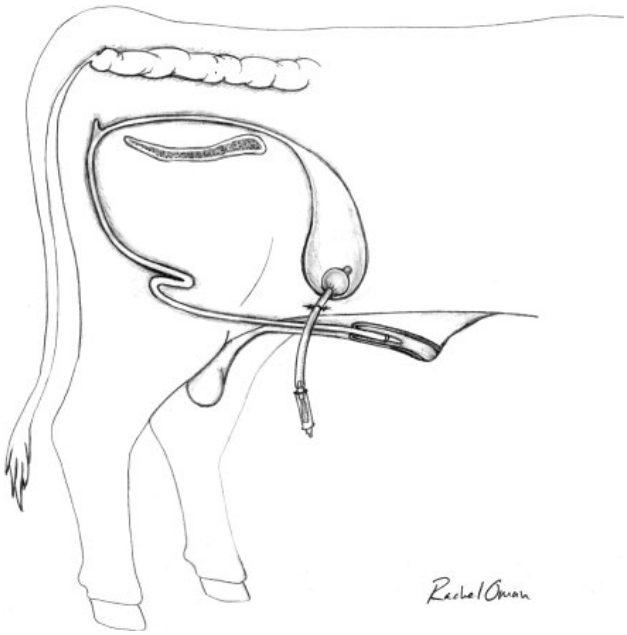


Figure 20.4 Placement of a Foley catheter in the tube cystotomy procedure. *Source:* Courtesy of Rachel Oman.



Figure 20.5 Placement of a Foley catheter in the flank tube cystotomy procedure.

For a paramedian tube cystostomy, general anesthesia is preferred, but a lumbosacral epidural and/or local anesthesia can also be utilized. The individual should be placed in dorsal recumbency and surgically prepped from sternum to pubis. If a uroabdomen exists, fluid stabilization and abdominal drainage should be performed prior to placement in dorsal recumbency. A 10- to 15-cm paramedian skin incision is performed approximately 2 cm lateral to the sheath approximately midway between the preputial orifice and pubis. A left-sided paramedian approach is preferred, so the rumen can assist with keeping abdominal viscera in the abdomen. If bladder rupture is suspected, the incision should be more caudal to assist with access to the smaller bladder often residing in the pelvis. Sharp and blunt dissection is used to access the linea alba and a routine celiotomy is performed. A paramedian celiotomy can be performed instead if preferred by the surgeon.

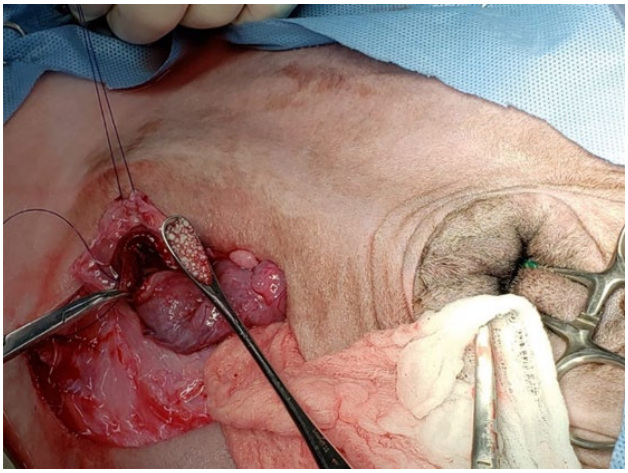


Figure 20.6 Utilization of stay sutures to assist with bladder manipulation and cystotomy. Utilization of a surgical spoon to assist with urolith removal.

Once the urinary bladder is localized, stay sutures with 2–0 or 0 monofilament can be placed in the bladder to assist with the cystotomy (Figure 20.6). A routine cystotomy is performed with aspiration of urine. Removal of uroliths from the bladder trigone and proximal urethra can be assisted with the use of a surgical spoon (Figure 20.6), as well as copious lavage and aspiration of sterile saline. An 8- to 10-French polypropylene catheter is then passed in a normograde fashion with hydropulsion to attempt resolution of the urethral obstruction. If the obstruction is not relieved or there is significant resistance, hydropulsion attempts should cease and the catheter should be removed. A stab incision is made 2 cm lateral to the paramedian skin through the skin and abdominal wall. A curved hemostat can be used intra-abdominally and directed toward the skin and incised over while placing pressure on the hemostat. The hemostat is used to grab the balloon end of a 20- to 30-French Foley catheter to assist with placement through the skin and abdominal wall. The balloon integrity of the Foley catheter should always be ensured prior to placement. Once the Foley catheter is in the abdomen, a separate stab incision is performed lateral to the primary cystotomy incision. A hemostat is placed through the stab incision of the bladder and the balloon end of the Foley is placed into the lumen of the bladder. The balloon is filled with the appropriate volume of saline.

Once filled, the balloon is directed toward the trigone of the bladder while the cystotomy incision is made and is sutured. A single- or double-layer continuous inverting pattern using absorbable monofilament 0 or 2–0 suture is used for the cystotomy and, if needed, cystorrhaphy. The balloon of the Foley catheter is pulled flush against the bladder and abdominal wall. Some surgeons choose to place a purse string suture around the Foley catheter [27]. The celiotomy, subcutaneous tissues, and skin are closed in a routine fashion. During closure, copious lavage with saline into the Foley catheter is important to prevent occlusion from any blood clots. A Chinese finger cuff ligature is used to secure the Foley to the ventral abdomen. After placement of the Chinese finger cuff ligature, saline should be flushed through the Foley catheter to ensure patency and confirm the ligature is not too tight.

A left flank tube cystotomy provides an economic alternative to tube cystotomy placement and avoids the risks associated with general anesthesia and dorsal recumbency. However, optimal access to the bladder is obtained through a ventral celiotomy. The left paralumbar fossa is aseptically prepped and flank anesthesia is performed. A 10- to 15-cm vertical skin incision is made with a routine celiotomy. Care should be taken to make the paralumbar fossa incision somewhat caudal to allow adequate access to the bladder. A needle and extension set can be used to drain excess urine from the bladder in particularly painful individuals attempting recumbency during surgery. The Foley catheter is placed through a stab incision through the skin and abdominal wall as described above on the caudoventral aspect of the incision. The balloon end of the Foley catheter is then placed through the apex of the bladder wall using a hemostat and bluntly forcing through the bladder wall. The balloon is inflated. If access is possible, a cystotomy should be performed on the dorsal aspect of the bladder to remove as many cystoliths as possible. Often a cystotomy is difficult with a standing procedure, so tube cystotomy alone can be performed, but recovery time will often be prolonged without removal of cystoliths. Closure of cystotomy or cystorrhaphy should be performed with 0 or 2–0 monofilament absorbable suture in an inverting pattern. The balloon of the Foley catheter is then pulled flush against the bladder and abdominal wall. A Chinese finger cuff ligature is used to secure the Foley catheter to the ventral flank. The abdomen and skin are closed in a routine fashion.

A one-way valve should be affixed to the Foley catheter to prevent air aspiration and minimize bacterial contamination. The fingertip of an examination glove with the tip slit affixed to the Foley catheter is a cheap, convenient one-way valve. After placement, a tube cystotomy must remain in place for a minimum of 7–10 days before removal to prevent any urine leakage from the defect created by the catheter. Urinary acidification should be implemented postoperatively for uroliths amenable to dissolution. Cystic acidification can also be administered with an acidifying solution seven days postoperatively with commercially available buffered acetic acid solution (Walpole's) or hemiacidrin (Renacidin®) [28]. A less expensive alternative for cystic irrigation is diluted acetic acid or 5% ammonium chloride solution [29]. Once urine dribbling from the preputial orifice or urination is observed, the Foley catheter can be occluded to more safely assess urethral patency prior to removal. One study reported dribbling of urine six to seven days postoperatively and free urine flow from the urethra nine days postoperatively [26]. The Foley catheter should be occluded for 48–72 hours with observed normal voiding before the Foley balloon is deflated and catheter removed. An average of 10–12 days postoperatively is required for small ruminants before successful voiding and Foley catheter removal [12, 23]. However, normal voiding can take as long as four to six weeks, especially if removal of cystoliths is not performed.

Complications of tube cystostomy include occlusion of the tube with blood clots, debris, uroliths, tube kinking, dislodgement or loss of catheter [26], balloon deflation, cystitis, uroperitoneum after catheter removal, adhesions to the urinary bladder, bladder atony, and failure to resolve urethral obstruction. Minimally invasive tube cystotomy [30] and laparoscopic-assisted placement [31] of cystic catheters have been described in small ruminants. Prognosis for survival in cattle treated with tube cystotomy is reported to be as high as 90% [18]. In cases without urethral rupture, utilization of a tube cystotomy provides a good treatment option for bulls to maintain breeding function.

Cystotomy

A cystotomy is ideally performed under general anesthesia with the individual in dorsal recumbency. Prior to creating the incision, the penis should be exteriorized and secured by clamping the apical ligament of the penis. A paramedian skin incision is performed lateral to the prepuce. The incision should be positioned relatively caudally to ensure adequate access and exteriorization of the bladder [27]. A paramedian or midline celiotomy can be performed. Once the urinary bladder is localized, stay sutures with 2–0 or 0 monofilament can be placed in the bladder to assist with the cystotomy. A routine cystotomy is performed with aspiration of urine. Removal of uroliths from the bladder trigone and proximal urethra can be assisted with the use of a surgical spoon, as well as copious lavage and aspiration of sterile saline. An 8- to 10-French polypropylene catheter is then passed in both a normograde and retrograde direction. Hydropulsion is performed with saline and may relieve the urethral obstruction. However, excessive force

and effort are contraindicated due to the risk of damaging the urothelium or causing a urethral rupture. The urethra is considered free of calculi when saline can easily be flushed normograde and retrograde [27]. If urethral patency is not obtained, a tube cystostomy should be performed. The bladder is closed with 2–0 or 3–0 monofilament suture in a single- or double-layer inverting suture pattern. In cases of bladder rupture, the defect should be repaired and/or any necrotic tissue removed. In cases of bladder rupture, aspiration and lavage of the abdomen should be performed. The celiotomy, subcutaneous tissues, and skin should be closed in a routine fashion. Disadvantages of this procedure include the expense and duration of the procedure, potential for urethral rupture, and extensive postoperative urethral swelling resulting in significant stranguria or postoperative obstruction.

Ischial Urethrostomy

Ischial urethrostomy is a viable treatment option for feedlot steers (≥318 kg) and breeding bulls [4, 32]. This procedure is performed under standing epidural anesthesia. The perineal region from the anus to the base of the scrotum is aseptically prepared. A 10-cm midline skin incision is performed starting over the ischial arch approximately 5 cm ventral to the anus [4]. The dense layer of fascia between the skin and retractor penis muscle is incised to expose the retractor penis muscle [4]. Blunt dissection between the retractor penis muscles reveals the bulbospongiosus muscle [4]. The urethrotomy incision is made through the bulbospongiosus muscle or just distal to its attachment [33]. Excessive hemorrhage is common during this procedure if the urethra incision is off midline and the corpus spongiosum is nicked. A 20- to 28-French Foley catheter is advanced into the bladder. Entrance into the bladder can be assisted with the use of a rigid stylet or curved hemostats to direct the tip over the ischial arch [4]. The Foley balloon is distended with saline and retracted until it is seated into the trigone of the bladder and sutured in place where it exits the skin. A one-way valve utilizing the fingertip of a glove with a slit should be taped onto the external end of the Foley catheter to prevent air aspiration into the bladder and minimize bacterial contamination. Ideally, impervious plastic should be adhered to the skin just ventral to the anus to prevent fecal contamination of the surgical site. For breeding bulls in which urethral patency is desired, a sterile bandage over the incision with stay sutures and surgical towels is recommended (Figure 20.7).

For feedlot steers, the catheter can remain in place until desired slaughter weight is reached or the uremia is resolved (~30 days) [32]. Additionally, this procedure can be used when a previous lower perineal urethrostomy site has strictured or reobstructed. In general, the prognosis is greatly reduced for individuals with bladder rupture. However, this technique allows the bladder to remain empty and heal by second intention.



Figure 20.7 Placement of a plastic or rubber (source-automobile inner tube) strip sutured to the perineum dorsal to the surgery site is recommended. *Source:* Image courtesy Dwight Wolfe and Misty Edmondson.

This technique can be a good option of urinary diversion for breeding bulls (without urethral rupture) because the urethral diameter is larger in this region, thus decreasing the risk of urethral stricture. If urethral patency is immediately obtained after the procedure, the Foley catheter can be removed and 3 mm polyethylene tubing is inserted through the urethrotomy incision into the bladder and exiting the distal urethra [4]. A primary urethral closure should be performed and the catheter exiting the distal urethra should be sutured to the ventral abdomen with a Chinese finger cuff ligature [4]. If urethra patency is not immediately obtained, the Foley catheter should remain in place to allow for relief of urethral spasm and swelling. If the obstruction fails to resolve after three to five days, alternative methods of calculi removal should be implemented. Other methods include retrograde catherization and hydropulsion, urethroscopy and possible basket retrieval, chemolysis (Walpole's solution, acetic acid, or hemiacidrin) for phosphatic uroliths (struvite or apatite), or urethrotomy. Long-term outcome and complication rates for breeding bulls have not been evaluated. However, there is one report of a goat with a strictured perineal urethrostomy being reversed with a buccal mucosal graft urethroplasty successfully [34].

Urethrotomy

Urethrotomy can remove individual uroliths or uroliths unamenable to dissolution with urinary acidification. This procedure is most utilized in breeding bulls since the urethra is not altered from its natural course. Additionally, this procedure is only a via option for individuals without urethral rupture. To perform the procedure, the urolith must be identified via palpation, catherization, or ultrasound. Depending on the location of the urolith, this can be performed under epidural anesthesia, general anesthesia, or sedation with casting harnesses. The individual is placed in dorsal recumbency, the urolith is located, and 2% lidocaine is infiltrated over the site of obstruction. The penis is grasped and a skin incision is performed. The penis is then exteriorized through the skin incision. A towel clamp or tissue forceps can be used to crush the stone and allow the fragments passage [4]. However, if this fails after two attempts, a urethrotomy should be performed directly over the urolith and the urolith(s) should be removed [4]. Some authors recommend bidirectional urethral flushing to ensure patency [21]. Urethral closure is then performed with 3–0 or 4–0 monofilament suture in a simple interrupted pattern. Suturing over a urethral catheter facilitates closure. The subcutaneous tissues and skin are closed in a routine fashion. Concurrent treatment with a urinary diversion technique such as tube cystostomy or ischial urethrostomy is ideal to allow adequate healing of the urethrotomy site. Urinary diversion allows resolution of inflammation at the urethra and thus prevents the risk of dehiscence or fistulation at the urethrotomy site. Alternatively, urethral catheterization can be used 24–48 hours postoperatively. Complication of urethrotomy includes urethral dehiscence and stricture especially if the urethral mucosa is devitalized. Peripenile adhesion is also a common complication that ultimate prevents a bull from reaching an erection and extension for breeding.

Perineal Urethrostomy

A perineal urethrostomy with penile amputation is a common salvage technique used for lightweight feedlot steers. This procedure is not intended for breeding individuals. Urethral stricture is an eventual complication of this technique; therefore it should be reserved for feedlot steers to resolve their azotemia and finish growth prior to slaughter. This technique is relatively quick and can easily be performed in the field.

It can be performed with the individual restrained in a chute under epidural anesthesia or in dorsal recumbency with heavy sedation and epidural anesthesia. The perineal area from the anus to the scrotum is clipped and aseptically prepped. A 10- to 15-cm midline incision is made immediately caudal to the scrotum. The incision is extended through the subcutaneous tissues and semitendinosus muscles. Blunt dissection is used until the penis can be grasped. The retractor penis muscles can often be confused for the penis. The retractor penis muscles are pink, soft, and identifiable as two separate components versus the firm penis which is covered in white tunica albuginea [21]. Once the penis is grasped, firm traction is used to pull the penis caudodorsally toward the skin incision, while the retractor penis muscles can be reflected or sharply dissected. Caudal traction and dissection are performed until a 6-cm segment of penis can be exteriorized without tension [21]. If substantial subcutaneous urine accumulation has occurred, the penis can separate from the preputial attachments, allowing exteriorization of the entire penis [4]. The dorsal penile artery and vein are identified and ligated approximately 5 cm distal to the dorsal aspect of the skin incision [21]. The penis is transected distal to this ligature. However, removal of the distal penis requires substantial dissection of the preputial attachments in cases with urethral obstruction but absence of urethral rupture. In these cases, the dorsal penile vasculature should remain intact and be blunted dissected and reflected from the penis.

Once the penis is transected, it is turned over 180° with the urethra now oriented dorsally. A horizontal mattress pattern using a monofilament non-absorbable suture is made through the corpus cavernosum to secure the penile stump to the skin [4, 21]. Caution should be used to not incorporate the urethra; a retrograde polypropylene catheter may be placed to ensure inadvertent urethral involvement is avoided (Figure 20.8). To assist with prevention of urine scald, the stump should extend 2–3-cm from the skin incision [21]. Spatulation of the urethra is optional (Figure 20.8). If performed, the urethra is incised from the distal aspect of the penile stump to the proximal aspect of the incision. The urethral mucosa and tunica albuginea are sutured to the skin using an absorbable 2–0 monofilament suture in a simple continuous or interrupted pattern. Spatulation of the urethra is performed to decrease the rapidity of urethral stricture formation [21]. The skin remaining proximal and distal to the urethrostomy site is closed in a routine fashion.

For individuals with a urethral rupture, subcutaneous skin incisions should be made as/if needed to drain any subcutaneous urine accumulation. Individuals with uroabdomen should have abdominal drainage of urine performed.

Potential complications from this procedure include postoperative hemorrhage from the corpus cavernosum of the penile stump and hemorrhage from repeated trauma to the penile stump. A securely fitted urethral catheter can be placed to put pressure on the corpus spongiosum to assist with hemorrhaging. Other common complications include urine scald, urethral stricture, and reobstruction with blood or calculi.



Figure 20.8 Perineal urethrostomy showing spatulation of the urethra and use of polypropylene catheter to assist with urethra location during suturing.

Other Procedures

Other treatment methods include laser lithotripsy, basket catheter urolith retrieval, and bladder marsupialization. Laser lithotripsy and basket catheter urolith retrieval will only be briefly mentioned here due to their limited use at referral institutions. Bladder marsupialization is only briefly mentioned due to the high complication rate in bovines.

Removal of urethroliths via laser lithotripsy has been reported in the literature for steers, goats, and pot-bellied pigs [35, 36]. An endoscope and laser fiber can be passed retrograde or normograde through an ischial urethrotomy [35]. Once the urethrolith is identified, the fiber is fired in a pulsatile fashion on the stone until fragmented enough to clear the urethra.

Basket catherization involves inserting a basket catheter into the urethral orifice and manipulating the instrument past the urethrolith. The instrument is opened, the urethrolith is maneuvered within the basket, and the catheter is removed. One report states a 55% successful urethrolith removal in calves [37] and another report successfully treated a bull with basket catherization while maintaining fertility [38].

Bladder marsupialization is often used in small ruminants as a last resort procedure with recurrent episodes of urethral obstruction. This procedure can also be utilized for detrusor muscle atony of the bladder. The apex of the bladder mucosa is sutured to the skin, allowing constant drainage of urine from the bladder. Numerous complications associated with this procedure include urine scald, bladder mucosa prolapse, stoma stricture, cystitis, and ascending pyelonephritis [12, 24, 25, 39]. It is not recommended for cattle. One study reported a mortality rate of 77% within two weeks of surgery [2]. Another study performed bladder marsupialization 12 hours after experimental induction of the urethral obstruction; of the six calves included in the study, one calf died and the remaining five calves had stoma stricture within three weeks postoperatively [40]. A novel procedure involving vesiculopreputial anastomosis has been described in small ruminants with a 50% success rate at six months postoperatively [41].

Prevention

The most important aspect of urolithiasis prevention is dietary modification. Ideally, the urolith(s) should be submitted to a laboratory for urolith analysis to determine composition. In the absence of this information, gross appearance of the urolith and ration evaluation should assist in figuring out the likely calculi involved. For phosphate calculi, excessive dietary phosphorus should be avoided, and rations should be adjusted to contain a calcium to phosphorus ratio of 1.5–2:1 and dietary magnesium should be less than 0.2%. Grain and alfalfa feeding should be reduced. For individuals fed a total mixed ration, roughage content should be evaluated. The primary excretion of phosphorus in ruminants is saliva, not urine. Therefore, if roughage is increased in the ration, rumination increases and salivary excretion of phosphorus is increased while solute in the urine is decreased. For silica uroliths, avoidance of at-risk pastures and feedstuffs is recommended. High-risk pastures can be used to graze female cattle instead of bulls and steers.

Adequate water consumption is critical in the prevention of urolithiasis. Ensuring access and availability to good quality water sources should be evaluated. If quality water sources are available, addition of sodium chloride to the ration should be considered to increase water consumption, ultimately creating dilute urine. According to Hawkins, sodium chloride has an effect of displacing magnesium and phosphorus from nucleation centers and thus preventing crystal development and urolith formation [1]. Silica uroliths form predominantly in the last 60 days before weaning; therefore a heavily salted creep feed is recommended for calves in the prevention of siliceous uroliths [4, 27, 42]. Sodium chloride can be fed up to 3–5% of the daily dry matter intake; this level of salt should be gradually increased to this level [4, 27].

Alternatively, reduction in urine pH can be attempted by feeding ammonium chloride at 0.5–1% of dry matter intake in a ration, which may help prevent apatite, struvite, and carbonate uroliths [27]. Feeding a DCAD ration can also be used to lower urinary pH in cattle. Silica urolith formation is not affected by urine pH. Caution should be used with long-term use; reduced bone mineral density has been reported in mature ewes fed anionic salts long term [43].

As previously discussed, urethral diameter is testosterone dependent. Delaying castration would result in a larger urethral diameter. According to Hawkins [1], a calf castrated at seven months of age could pass a urolith 13% greater in diameter and a bull could pass a urolith 44% greater in diameter than a calf castrated when one month old. While the evidence exists that delaying castration increases urethral diameter, the welfare consequences of delaying castration should be considered prior to implementation. Dietary modification and water consumption would be better areas of focus for prevention.

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21

Preparation of Teaser Bulls

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Introduction

Historically, a key to a successful artificial insemination (AI) program has been accurate detection of estrus. Even with utilization of currently available estrus synchronization programs and timed AI, estrus detection is important as a tool to evaluate the efficacy of the protocol, troubleshoot problems in real time, and identify outliers that can be bred outside the prescribed "AI window."

Several estrus detection methods exist, including tailhead paint, mount detectors, self-adhesive heat detection patches, and visual observation. All these methods depend on female cattle standing for mounting during estrus. Thus these methods might miss females with weak or short estrus behavior [1]. By far the most efficient estrus detector is the bull, with the caveat that there is the benefit of male presence [2]. Therefore utilization of a teaser bull (intact sterilized male) is the most reliable method of estrus detection in the utilization of an AI program.

Several factors need to be considered when choosing a teaser bull procedure and each producer will have different needs and expectations. Besides the obvious need to render the bull sterile, other considerations would include herd status (open or closed herd). If an open herd, venereal disease transmission is an important factor to consider and prevention of intromission during mounting will be an important factor when choosing a teaser bull surgical procedure. Additionally, expected longevity needs to be discussed with the producer. On average, teaser bulls will last one to three years within a herd [1]. Decreased libido is the most common reason for culling, with excessive size and aggression being the next most common culling reasons [3]. To summarize, the main goals of surgical preparation of teaser bulls are to render him sterile, prevent intromission and therefore the transmission of venereal disease, and avoid excessive libido reduction [4, 5].

Proper bull selection is also an important aspect of teaser bull preparation. The ideal bull needs to be of moderate size, mild temperament, and easily handled. This bull also needs to be free of transmissible diseases. Of course, the bull also needs strong libido, but this can be difficult to assess in yearling bulls [4]. Teaser bull surgery should be performed well before the breeding season to allot time for healing and recovery from surgery. Ideally, the procedure should be performed on bulls less than 272 kg, primarily for ease of handling and decreased hemorrhage during surgery [4].

Teaser bull procedures can be divided into two categories: those that block semen flow and deliver sterility (vasectomy, epididymectomy) and those that prevent penile penetration (penile–prepuce translocation, penopexy, preputial pouch). Depending on the needs and expectations of the producer, any one or a combination of these procedures can be used for preparation of a teaser bull.

Vasectomy

As previously mentioned, vasectomy will render a bull sterile but does not prevent normal mating and copulation behavior. This procedure can be performed with the bull in standing restraint or recumbency, or a tilt chute may be used if available. The typical surgical approach is an anterior approach on the neck of the scrotum. However, if standing restraint is chosen, then the approach would be the posterior aspect of the neck of the scrotum. Figure 21.1 shows the procedure.

The neck of the scrotum should be clipped and aseptically prepared for surgery. Lidocaine 2% should be infused over the proposed incision site over each spermatic cord. A 3-cm incision should be made through the skin and tunica dartos over each spermatic cord. The spermatic cord is isolated by placing a hemostat underneath the entire spermatic cord.

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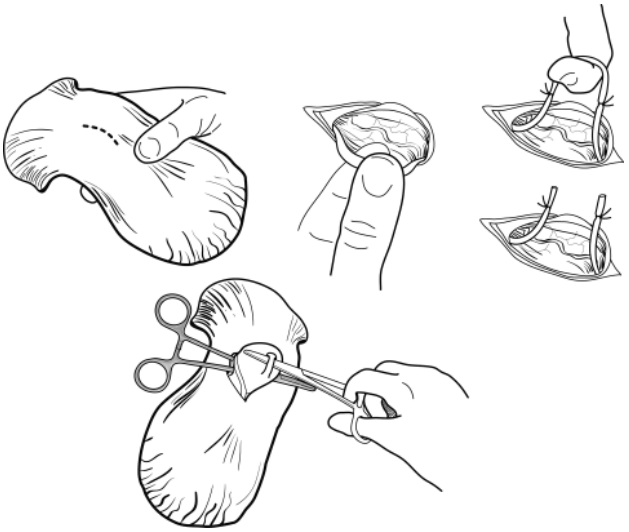


Figure 21.1 Procedure for vasectomy. *Source:* Illustration by Mal Hoover.

The ductus deferens is then identified via palpation. The ductus deferens is a firm structure that runs medially along the spermatic cord and is approximately 2–3 mm in diameter. Once identified, the tunica vaginalis is carefully incised, utilizing extreme caution so as not to damage the cremaster muscle or pampiniform plexus resulting in excessive hemorrhage. After the tunica vaginalis is incised, the ductus deferens is isolated with another hemostat.

Two ligatures are placed approximately 3–5 mm apart using #0 absorbable suture [4, 5]. The ductus deferens is removed between the two ligatures. The skin is closed with a cruciate pattern using nonabsorbable suture. Antibiotics can be administered to prevent any postoperative infection, especially if surgical contamination has occurred. It is recommended to wait 30 days prior to using the bull as a teaser animal, since sperm can be present in the reproductive tract up to 30 days postoperatively [4, 5]. Additionally, it is recommended to perform yearly evaluations of the teaser animal's ejaculate to ensure sterility of the animal.

Epididymectomy

An epididymectomy is similar to a vasectomy with regard to restraint options and copulation behavior [6]. For this procedure, the base of the scrotum is clipped and aseptically prepared. Lidocaine 2% is infused over the tail of the epididymis. Once prepared, the surgeon grasps the neck of the scrotum and pushes the testicle ventrally. A 3-cm incision is made over the tail of the epididymis through the skin and common vaginal tunic until the epididymis is exteriorized. The tail of the epididymis is carefully dissected from the testicle, and towel clamps or Allis tissue forceps can be used to assist in handling and manipulation of the epididymis. A hemostat is placed on the ductus deferens and the body of the epididymis. Ligatures with #0 absorbable suture are placed proximal to the hemostats. The tail of the epididymis is removed by transection distal to the hemostats. Figure 21.2 shows the procedure.

The common vaginal tunic is closed using #0 absorbable suture. The skin can be closed with non-absorbable cruciate sutures or the incisions can be left open to allow ventral drainage. Antibiotics can be administered to prevent postoperative infections. Postoperative resting recommendations and yearly ejaculate examinations are the same as previously stated for vasectomy aftercare.

Penile–Prepuce Translocation

Penile–prepuce translocation ("sidewinder") is the surgical transposition of the penis and prepuce from the ventral midline to the right or left flank of a bull. This procedure allows normal protrusion and erection but does not permit intromission. In general, "sidewinders" are preferred by producers due to longevity and herd retention of the teaser animal. Bulls with a penile–prepuce translocation maintain better and longer libido since this procedure allows normal protrusion and does not cause pain during erection. Some bulls are able to compensate and learn how to breed females despite the translocation of the penis and prepuce. Therefore it is recommended that a vasectomy or epididymectomy is performed to ensure sterility of the bull.

Penile–prepuce translocation is performed in lateral recumbency, so general anesthesia is the preferred method of restraint. If general anesthesia is not possible, heavy sedation with rope restraints and local infiltration of 2% lidocaine can be used. Ideally, food should be withheld for 24 hours and water for 12 hours before performing the procedure.

Prior to placing the bull in recumbency, the translocation site for the preputial orifice should be identified. The translocation site should be just outside the flank fold and lateral to the original preputial orifice site [3]. An 18-gauge needle can be used to abrade the epidermis so the location is not altered after placing the animal in recumbency and skin stretching occurs. The ventral abdomen from the umbilicus to just cranial to the scrotum and the site of translocation of the flank should be clipped and aseptically prepared. Flush the prepuce with dilute iodine solution.

Before making the initial incision, place one simple interrupted suture at the dorsal aspect of the preputial orifice to serve as a marker and prevent twisting of the prepuce during translocation. A circumferential skin incision around the preputial orifice is made 4 cm from the orifice or a total diameter of 8–10 cm (Figure 21.3) [3, 4]. Extend

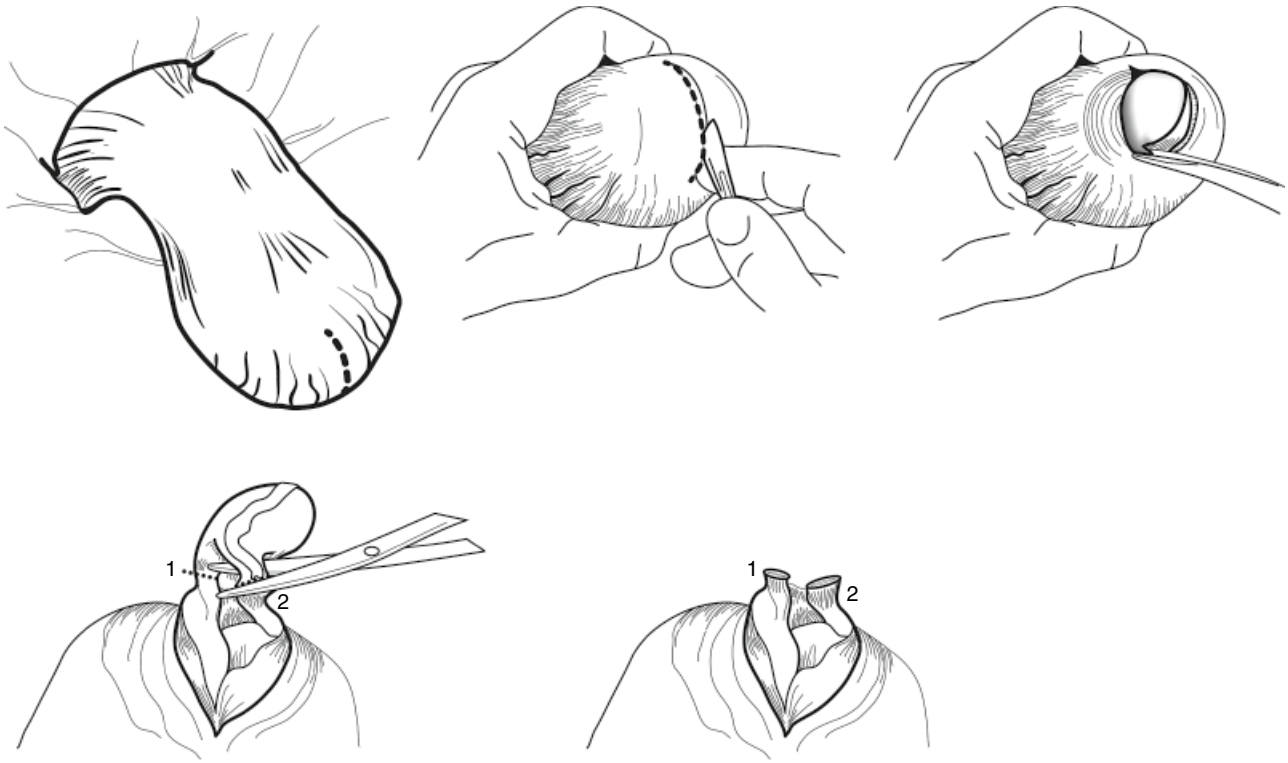


Figure 21.2 Procedure for epididymectomy. *Source:* Illustration by Mal Hoover.



Figure 21.3 Circumferential incision 4 cm from the preputial orifice is performed with an interrupted suture placed at the dorsal aspect of the preputial orifice to prevent twisting during translocation. *Source:* Photo courtesy of Tom Thompson.



Figure 21.4 Ventral midline incision extending caudally with circumferential incision at the translocation site. *Source:* Photo courtesy of Tom Thompson.

the skin incision on the ventral midline from the preputial orifice to just cranial to the scrotum (Figure 21.4). Carefully dissect the penis and prepuce from the ventral abdomen. Avoid lacerating the prepuce; packing or tubing can be placed in the prepuce to aid with proper identification. While dissecting the penis and prepuce, avoid incising the dorsal penile vessels and control hemorrhage as it is encountered. Once the penis and prepuce are dissected, make a circular skin incision equivalent to the diameter of the preputial orifice at the desired translocation site (Figure 21.4). Use a sponge forceps to create a tunnel toward the flank incision. As the forceps is retracted, open it slightly to help facilitate penile translocation. This tunnel can also be accomplished with a cold sterilized polyvinyl chloride (PVC) pipe (Figure 21.5).

Place a sterile glove or sleeve over the preputial orifice to minimize contamination of the subcutaneous tissues. Then run a sponge forceps from the flank incision to the ventral midline incision and grasp the preputial orifice.



Figure 21.5 Use of a cold sterilized PVC pipe to facilitate tunneling of penile translocation and skin incision for the translocation site. *Source:* Photo courtesy of Tom Thompson.



Figure 21.6 Closure of new preputial orifice with interrupted sutures and ventral midline with Ford interlocking pattern. *Source:* Photo courtesy of Tom Thompson.

Manipulate the preputial orifice to the flank incision, taking care not to twist the prepuce (use a stay suture to ensure proper alignment). Suture the skin around the preputial orifice using #3 non-absorbable sutures with a cruciate or horizontal mattress pattern (Figure 21.6) [1]. Close the subcutaneous layer of the ventral midline incision with #3 absorbable suture, closing as much dead space as possible to prevent seroma formation. Close the skin with #3 non-absorbable suture in a Ford interlocking pattern. Place a cruciate suture at the cranial aspect of the incision to be removed for drainage if a seroma does occur.

The teaser bull should be monitored closely for 24 hours postoperatively to ensure he is able to urinate properly. Antibiotics should be administered for three to five days postoperatively to prevent infection. Allow four to six weeks of recovery time before using the teaser bull [3–5]. Penile–prepuce translocation is not a technically difficult procedure, but it is more invasive and can result in more postoperative complications. The most common complications are obviously seroma and abscess formation from the excessive dead space created. Another complication would be not translocating the preputial orifice high enough on the flank and thus the bull would still be capable of breeding a female animal. There is also one case report of a teaser bull developing paraphimosis with a penile–prepuce translocation [7].

Penopexy

Penopexy is the iatrogenic creation of phimosis by surgically creating an adhesion of the penis to the ventral body wall. This procedure prevents protrusion of the penis, thus preventing normal intromission or copulation. Penopexy is a relatively quick procedure and can typically be performed with sedation and local infiltration of 2% lidocaine. Tilt chute restraint or general anesthesia can also be utilized. Lateral recumbency is the preferred positioning.

The bull's ventral abdomen is clipped and surgically prepared from the preputial orifice to the scrotum. A skin incision is made 2–3 cm lateral of the midline and half the distance between the preputial orifice and scrotum approximately 10 cm in length. Carefully dissect the subcutaneous tissues until the penis is identified and exteriorized (Figure 21.7). Once the penis is exteriorized through the incision, identify the caudal reflection of the penis (fornix) and dissect the subcutaneous tissues on the dorsal aspect of the penis until the tunica albuginea is exposed for approximately 10 cm caudal to the fornix [4, 5]. Remove the subcutaneous tissue on the linea alba in conjunction with the dorsal aspect of the penis. The tunica albuginea and corresponding linea alba are scarified to promote strong adhesion formation. After preparation of both sites, the urethral groove is identified on the ventral aspect of the penis. Beginning 6–8 cm caudal to the fornix of the penis, pre-place four to six simple interrupted sutures approximately 2 cm apart using a heavy non-absorbable suture [4, 5]. The suture is placed through the dorsal third of the penis using care to not enter the urethra. The suture is then placed through a corresponding area of the linea alba (Figure 21.8) [4, 5]. Once all the sutures are pre-placed, return the penis to the normal anatomical position and ensure it is not protruding from the preputial orifice prior to securing all the sutures (Figure 21.9). Close the subcutaneous tissue with absorbable sutures and the skin with #3 non-absorbable suture in a Ford interlocking pattern. A vasectomy or epididymectomy is usually performed in conjunction with a penopexy to ensure sterility of the bull in case of procedure failure.

Allow three to four weeks of recovery to ensure proper formation of adhesions. The drawback of penopexy is the

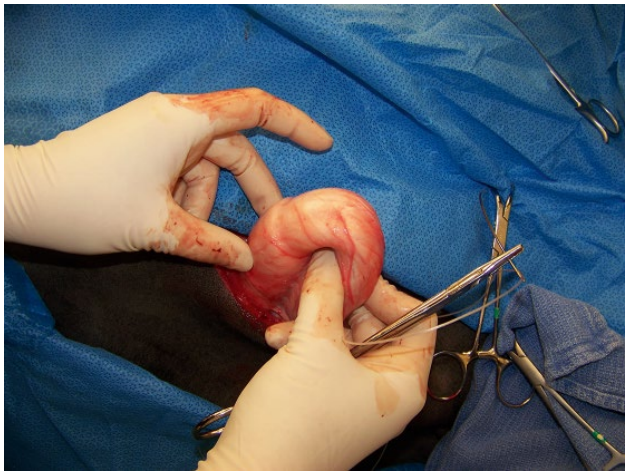


Figure 21.7 Exteriorization of the penis through the incision and identification of the caudal reflection of the penis.

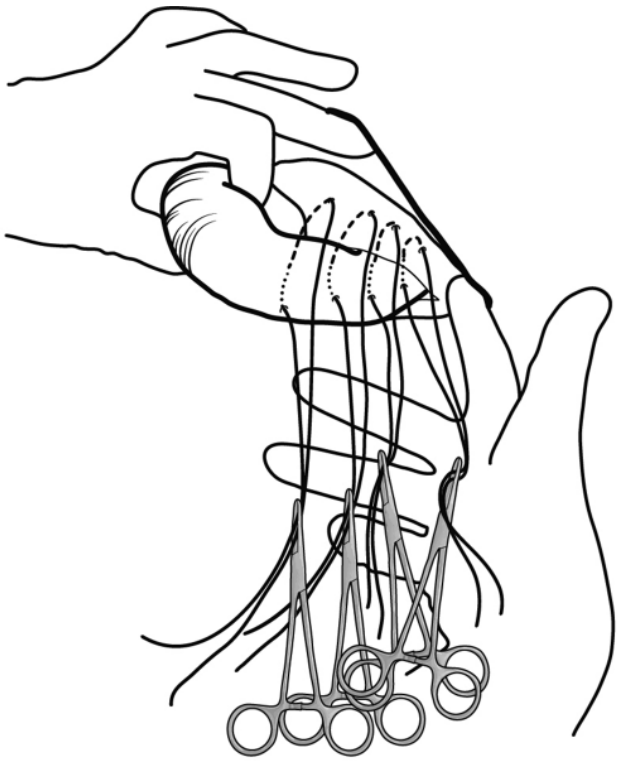


Figure 21.8 Preplacement of sutures through the dorsal third of the penis and linea alba. *Source:* Illustration by Mal Hoover.

risk of entering the urethra and decreased longevity in the herd since the bull will experience pain during attempted erection, thus decreasing libido. A follow-up study of 37 bulls found that 15% of bulls maintained good libido for one breeding, 30% for 1–1.5 years, and 42% for more than 1.5 years [5, 8].

A standing perineal penopexy approach has been described using light sedation and a caudal epidural. The

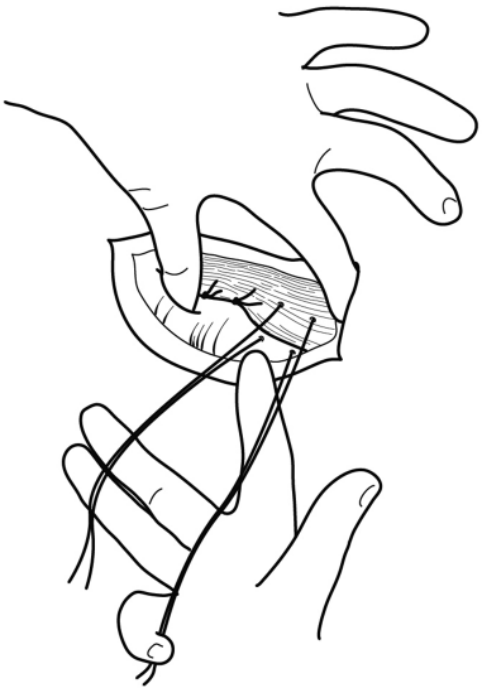


Figure 21.9 Securing the stay sutures for penopexy. *Source:* Illustration by Mal Hoover.

approach is over the distal loop of the sigmoid flexure [4]. A 4- to 5-cm incision is made through the skin and the tunica albuginea is exposed as mentioned previously. However, the stay sutures are placed on the lateral aspects of the penis and secured to the fibrous connective tissue in the perineal region of the bull [4, 5].

Preputial Pouch Technique (Ventral Slot with Preputial Orifice Obliteration)

The preputial pouch technique creates a fistula on the ventral prepuce and closes the normal preputial orifice. This technique prevents penile extension but allows for passage of urine through the ventral fistula. Teaser bulls with a preputial pouch are typically retained in the herd longer because libido is maintained for longer due to the lack of pain during attempted breeding [5]. This procedure is performed in lateral recumbency and can be accomplished with tilt chute restraint or sedation with local infiltration of 2% lidocaine. The ventral abdomen is clipped and prepared from the umbilicus to the midsheath region of the bull.

Prior to initiating surgery, the penis is extended and a Penrose drain is sutured around the glans penis with 2–0 polydioxanone (PDS). An approximately 1-cm-diameter elliptical incision is made through the skin 7 cm caudal to

the preputial orifice (Figure 21.10). The skin incision is extended through the preputial mucosa. The excised skin and mucosa are discarded. Then the internal mucosa of the prepuce is sutured to the skin of the sheath to create the fistula. An interrupted non-absorbable suture pattern is recommended (Figure 21.11a and b) [5]. Once suturing is complete, the free end of the Penrose drain is placed through the fistula. The Penrose drain will facilitate urine divergence while the primary incision sites heal.

The preputial orifice obliteration is accomplished by removing approximately 5 mm of the sheath skin and prepuce mucosal junction around the entire preputial orifice (Figure 21.12). This incision is closed in three layers: preputial epithelium, subcutaneous layer, and skin. The Penrose drain and sutures can be removed in two weeks. Allow three weeks of postoperative recovery time prior to utilizing the teaser bull [5].

The critical step in the preputial pouch technique is the size of the fistula. If the fistula is too small, proper urine flow is obstructed. If the fistula is too large, penile extension can occur with potential breeding. Therefore it is recommended to perform a vasectomy or epididymectomy to ensure sterility of the bull. Some bulls may pool urine in their preputial pouch and require postoperative flushing of the pouch. Thus this technique is not recommended for *Bos indicus* breeds since their pendulous sheath would predispose them to urine pooling and calculi formation [5].

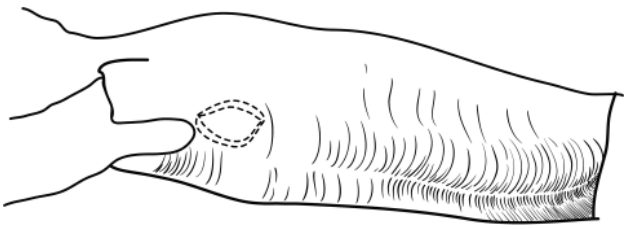


Figure 21.10 Site for incision for ventral fistula. *Source:* Illustration by Mal Hoover.

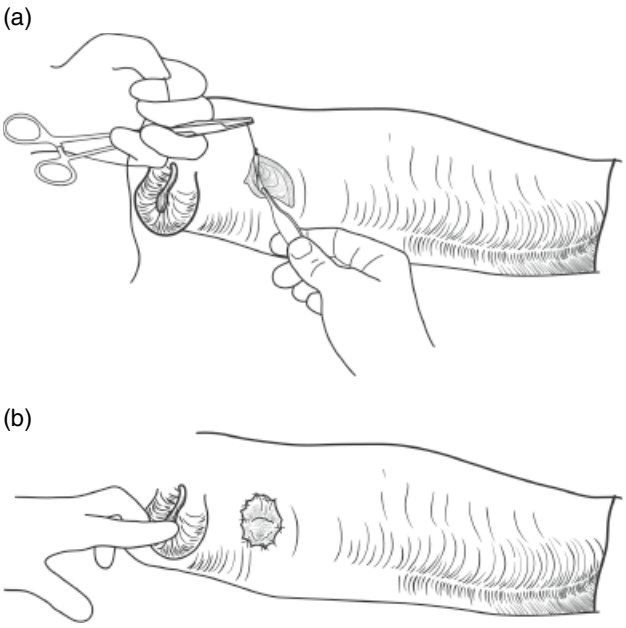


Figure 21.11 (a and b) Suturing of preputial mucosa to the sheath skin. *Source:* Illustration by Mal Hoover.

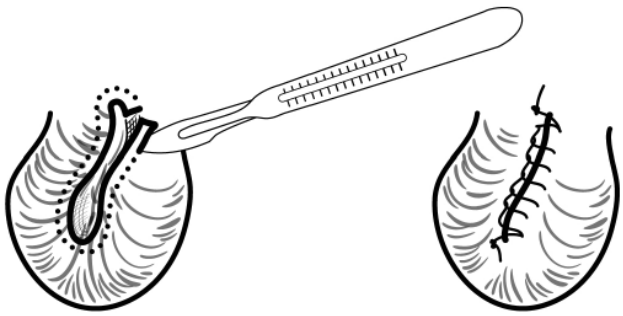


Figure 21.12 Excision of 5 mm of the preputial epithelium and sheath skin junction. *Source:* Illustration by Mal Hoover.

Other Procedures

Other teaser bull procedures include iatrogenic preputial stenosis, artificial corpus cavernosal thrombosis, transection of the apical ligament, and penectomy. These procedures are briefly discussed, since their use has fallen out of favor due to high risk of failure, complication rates, and diminished libido.

Iatrogenic preputial stenosis involves a ventral mid-sheath approach to the prepuce and penis. The prepuce is identified and a stainless-steel rod or Steinmann pin is secured and tightened around the prepuce. Care must be taken to tighten the ring sufficiently to prevent penile extension, but loose enough to prevent urine pooling and balanoposthitis [5, 9]. Mixed success rates accompany this procedure. Complications associated with this procedure include excessive ring closure resulting in urine retention and balanoposthitis, complete stenosis resulting in subcutaneous urine accumulation, lack of stenosis resulting in penile extension and intromission, and excessive tissue reaction to the stainless-steel ring.

Corpus cavernosal thrombosis involves injection of an acrylic material into the corpus cavernosum of the penis [10]. The acrylic material results in thrombus formation that prevents erection. This method is performed with standing restraint under a caudal epidural. A midline incision is made over the penis. The distal sigmoid flexure is identified by locating the retractor penis muscle, with the proximal sigmoid flexure being approximately 15 cm proxi-

mally. A 14-gauge needle is inserted at the dorsolateral aspect of the penis at the proximal sigmoid flexure and the acrylic is injected. Non-absorbable stay sutures are placed at the lateral aspect of the penis at the level of the retractor penis muscle to prevent penile prolapse. Potential complications of this procedure include inadequate injection of acrylic into the corpus cavernosum resulting in procedure failure, or accidental injection into the corpus spongiosum or urethra resulting in urethral obstruction [5].

Transection of the apical ligament involves intentional transection of the apical ligament of the penis creating a ventral penile deviation and preventing intromission. The bull is restrained in lateral recumbency, either with heavy sedation and rope restraint or utilization of a tilt table. The penis is extended and a towel clamp is placed around the apical ligament of the penis to maintain penile extension. Prepare the penis and prepuce aseptically. Just proximal to the clamp, 2% lidocaine is infused subcutaneously under the epidermis of the penis. An approximately 2-cm skin incision is made longitudinally along the dorsum of the penis. Once the apical ligament of the penis is isolated, the ligament is transected extending to the tunica albuginea. The skin incision is closed with interrupted absorbable suture. Possible complications of this procedure include excessive hemorrhage with secondary seroma or abscess formation. Additionally, inadequate transection of the apical ligament could occur or healing of the ligament could allow breeding occurrence, so a vasectomy or epididymectomy is recommended in conjunction with this procedure.

Penectomy involves amputation of the penis. This can be performed by amputation of the glans penis at the fornix and suturing of the prepuce to the urethral mucosa [11]; alternatively this can be performed at the perineal region, suturing urethral mucosa to the skin [5, 11]. Amputation of the glans penis at the fornix results in teaser bulls that experience pain during breeding attempts, thus decreasing libido and herd retention time [5]. With penectomy via the perineal approach, bulls often lose interest and experience decreased libido due to the lack of coitus [11]. With either approach, urethral stricture is a risk factor.

Summary

Accurate heat detection is essential to any AI or embryo transfer program and teaser bulls are the best at detecting heat. There are multiple procedures for creating a teaser bull, with no single procedure being perfect. Each procedure has its advantages and disadvantages. Ultimately, the decision of which procedure to perform depends on the needs and expectations of the client (longevity of bull, postoperative complications, assured sterility). Additional factors that may impact procedural choice include facilities, veterinarian preference, cost, and herd status.

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Section II

The Cow

Anatomy and Physiology

22

Anatomy of the Reproductive System of the Cow

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Introduction

The anatomy of the reproductive system in the cow is functionally grouped into the components associated with oocyte production and transport and those involved with gestation and copulation.

Production

The cellular machinery for oogenesis and steroid production is found in the ovary (Figure 22.1). The ovary consists of a cortex and medulla. The medulla is composed of connective tissue, lymphatic vessels, blood vessels, and nerves. Surrounding the medulla is the cortex. The cortex contains the ova surrounded by follicular cells within the connective tissue stroma [1]. Exterior to the cortex, the ovary is covered by the dense fibrous tunica albuginea and a superficial epithelium [2].

Because the ovary in the cow descends further from its embryologic origin near the kidney than other species, it is positioned closer to the pelvis. The consequence of this ovarian location and the attachment of the short mesovarium is that the uterine horns bend ventrally and caudally [3] (Figure 22.2). (Editor's note: for additional histological images of the ovary see Chapter 25.)

Transport and Gestation

The reproductive system of the cow is designed to transport spermatozoa toward the ovary and to transport an ovum toward the spermatozoa (Figure 22.3). The parts of this tubular system include the vestibule, vagina, cervix, uterine horns, and uterine tubes (oviduct).

Uterine Tube

The uterine tube is arranged like a funnel near the ovary. The funnel-shaped end, or infundibulum, contains processes, the fimbriae, which collect the ovum on ovulation (Figure 22.4). The ovum is then transported through the abdominal opening of the uterine tube located at the base of the infundibulum [4]. The ampulla of the uterine tube is the region adjacent to the infundibulum where fertilization takes place. The isthmus, the continuation of the uterine tube from the ampulla toward the uterus, is relatively long due to the meandering course it takes before ending at the uterine opening where it releases the ovum into the uterine horn [4].

Uterus

The uterus consists of a body and two horns (Figure 22.5). The body is short, beginning immediately after the cervix ends. The horns branch from the body but are joined together by the peritoneum, giving the appearance that the body is longer than it truly is. As the horns progress craniad they divide at the intercornual ligaments, each turning abruptly ventrally, then proceeding caudally, and finally ending dorsal to the ovary [3] (Figure 22.6).

From external to internal, the uterus can be divided into three layers: the perimetrium, the myometrium, and the endometrium [3]. The perimetrium is the continuation of the abdominal peritoneum onto the uterus. The myometrium constitutes the muscular layers, which can undergo substantial hypertrophy [5]. The endometrium is the internal epithelial lining of the uterus and is arranged into two distinct regions, caruncular and intercaruncular [6]. The caruncles are raised mucosal regions of the endometrium that are highly vascularized (Figure 22.7). The caruncles of the uterus join with the cotyledons of the fetal placental membranes to form the placentomes of a cotyledonary placenta [4].

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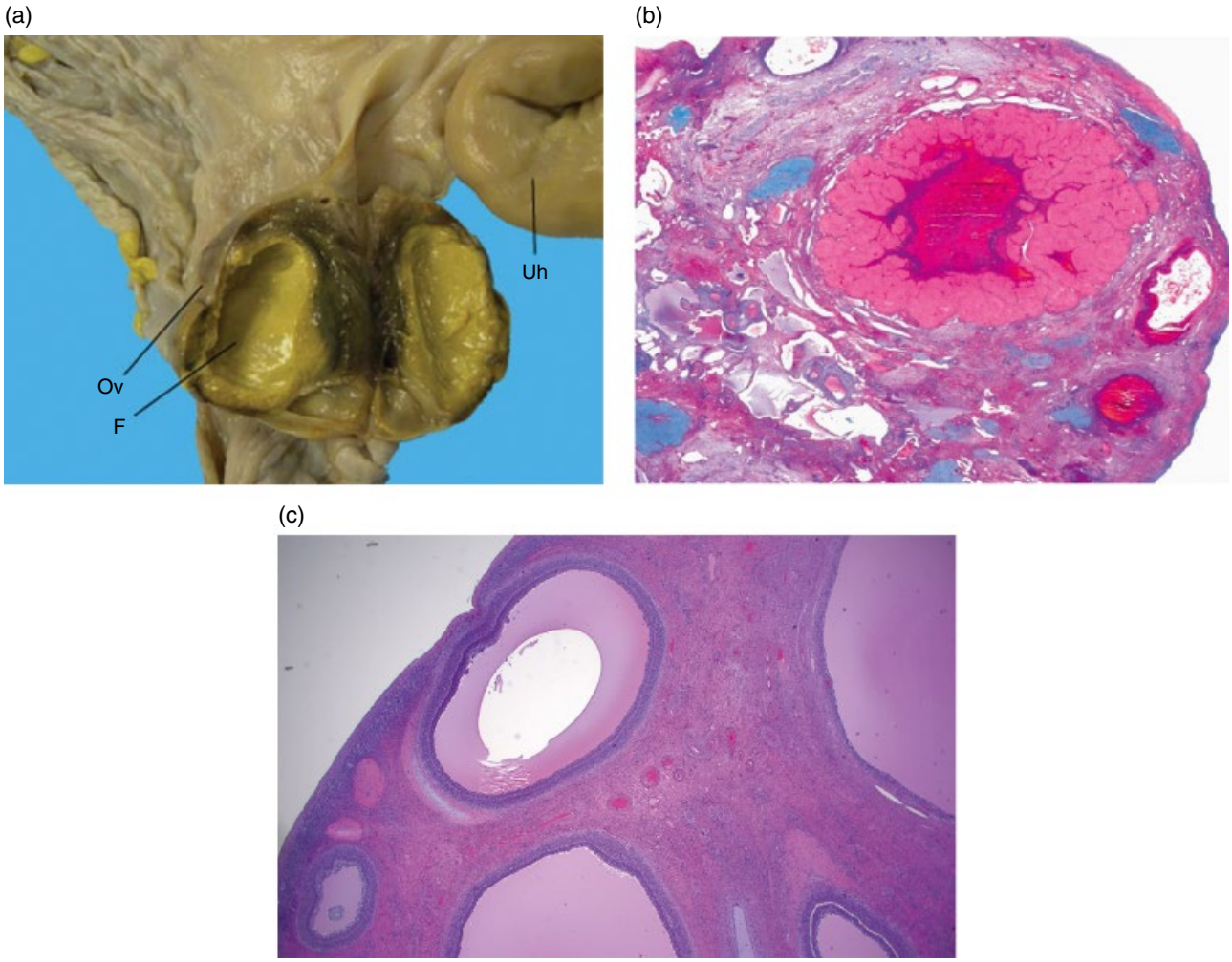


Figure 22.1 (a) Ov, ovary, Uh, uterine horn, F, follicle, 22.1 (b) Corpus luteum, Image courtesy Dr. Fuller Bazer, Texas A & M, (c) Corpus lutea (image from pregnant cow), Image courtesy Dr. John Roberts, College of Veterinary Medicine, University of Florida.

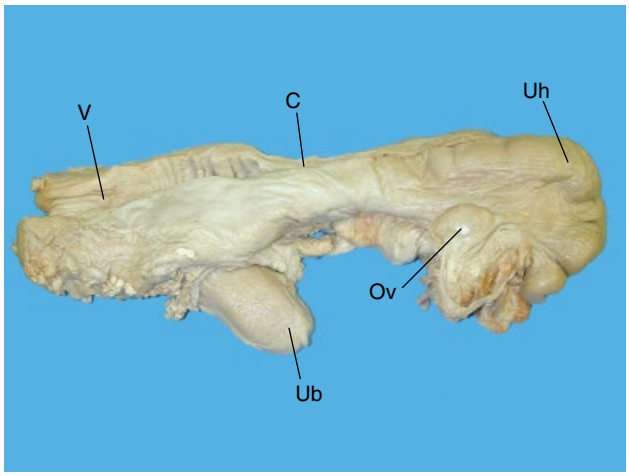


Figure 22.2 The internal reproductive tract from oblique angle. V, Vagina; C, cervix; Uh, uterine horn; Ov, ovary; Ub, urinary bladder.

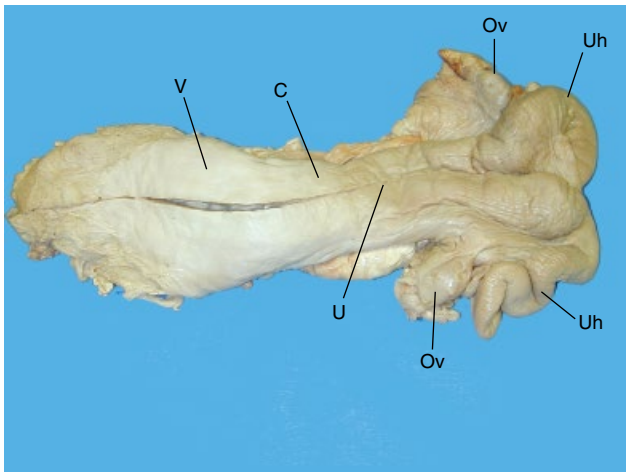


Figure 22.3 The internal reproductive tract from dorsal view. V, Vagina; C, cervix; U, uterus body; Ov, ovary; Uh, uterine horn.

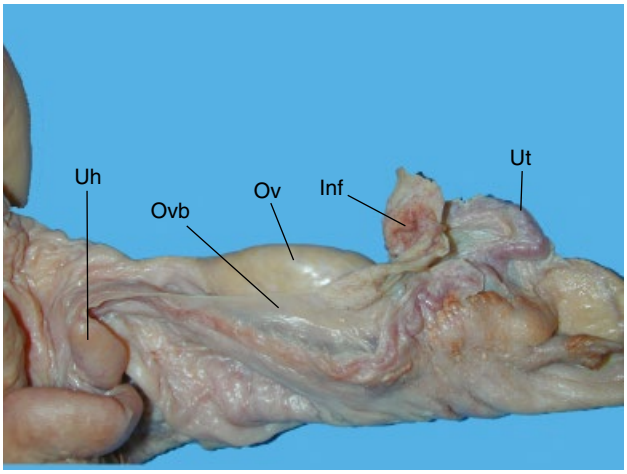


Figure 22.4 Structures of the ovary. Ov, Ovary; Inf, infundibulum; Ut, uterine tube; Ovb, ovarian bursa; Uh, uterine horn.

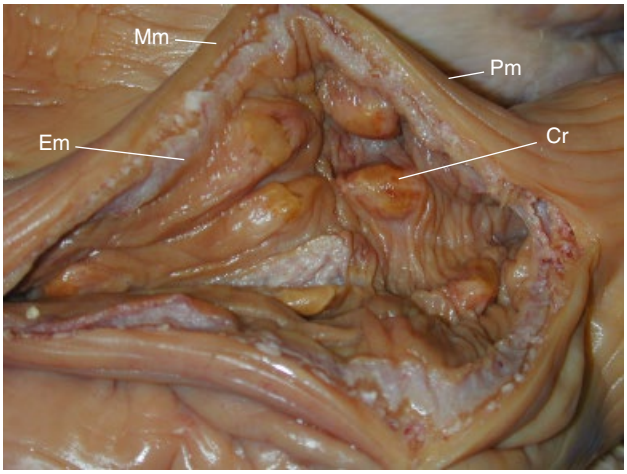


Figure 22.7 The uterine lumen. Pm, Perimetrium; Mm, myometrium; Em, endometrium; Cr, caruncle.

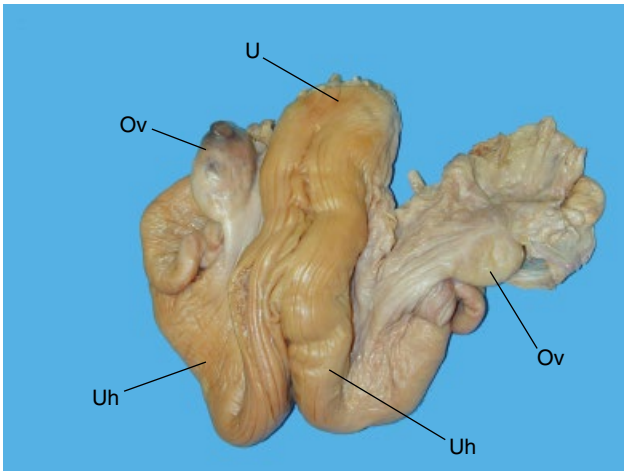


Figure 22.5 Dorsal view of reproductive tract. U, Uterus body; Ov, ovary; Uh, uterine horn.

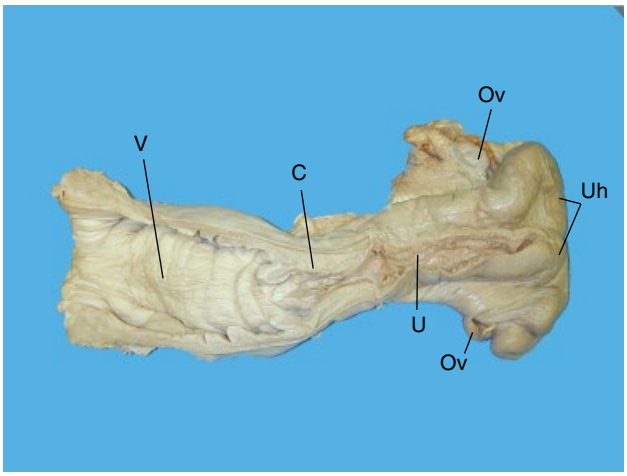


Figure 22.8 Vaginal and cervical lumen. V, Vagina; C, cervix; U, body uterus; Uh, uterine horn; Ov, ovary.

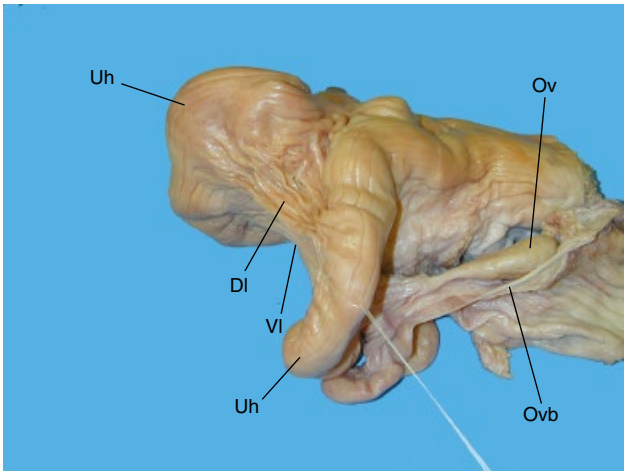


Figure 22.6 Uterine horns positioned to better view the intercornual ligament. Uh, Uterine horn; Dl, dorsal intercornual ligament; Vl, ventral intercornual ligament; Ovb, ovarian bursa; Ov, ovary.

Cervix

The cervix is located between the body of the uterus cranially and the vagina caudally (Figure 22.8). It is a firm, muscular, sphincter-like structure that acts as a barrier separating the external genitalia from the internal genitalia [5]. Characteristic of the cervix are the three to four circular folds projecting into its lumen.

The arrangement of the cervical musculature and mucosa is responsible for this characteristic architecture (Figure 22.9). The superficial mucosa is arranged in longitudinal folds punctuated by circular folds that interdigitate to form a series of ridges and interlocking notches when the cervix is closed [4]. This arrangement effectively seals the external environment from the internal uterine environment.

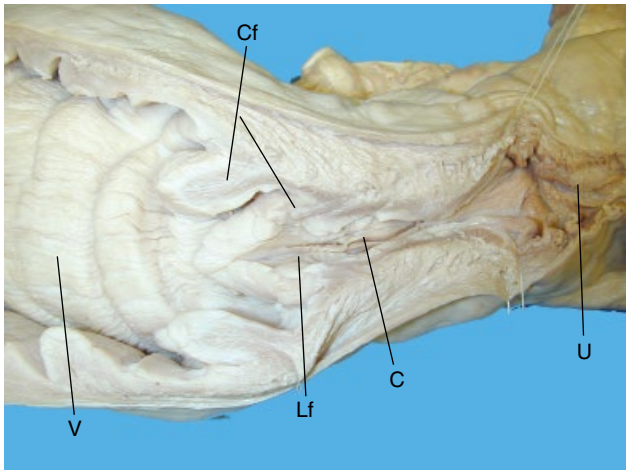


Figure 22.9 Close-up of vaginal and cervical lumen allowing visualization of the external cervical os. V, Vagina; C, cervix; U, uterus; Cf, circular folds; Lf, longitudinal folds.

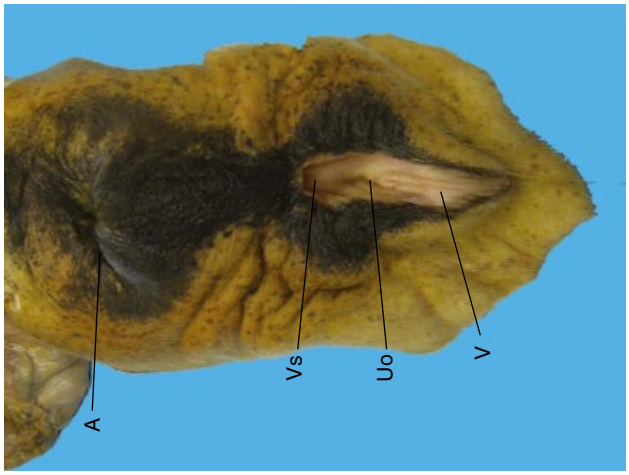


Figure 22.10 External view of perineum. A, Anus; Vs, vestibule; Uo, urethral orifice; V, vulva.

Vagina

The vagina is positioned between the caudal extent of the cervix and border of the vestibule at the external urethral orifice. The cervix projects into the lumen of the vagina caudoventrally, causing the dorsal vaginal fornix to form a deeper recess than the ventral fornix [4].

Vestibule

The vestibule is a small area in the cow that originates at the urethral opening and ends caudally to blend with the labia of the vulva.

Vulva

The labia of the vulva are located on either side of the labial fissure [3] (Figure 22.10). The labia meet dorsally, forming the dorsal commissure, and again ventrally to form the ventral commissure. The clitoris is found just cranial to the ventral commissure [3].

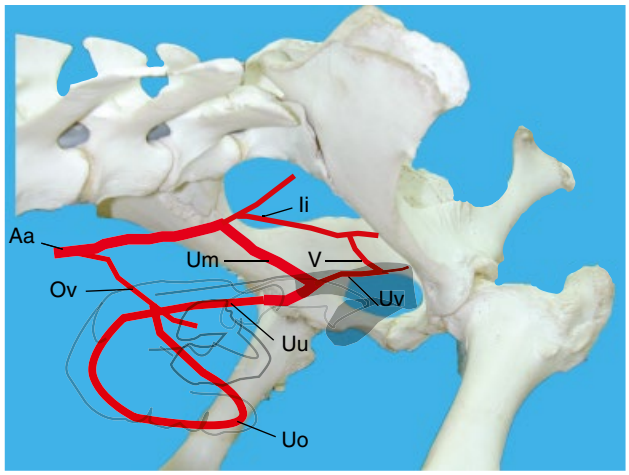


Figure 22.11 Arterial blood supply to the reproductive tract. Aa, Abdominal aorta; Ov, ovarian artery; Um, umbilical artery; Ii, internal iliac artery; V, vaginal artery; Uv, uterine branch of vaginal artery; Uu, uterine branch of the umbilical artery; Uo, uterine branch of ovarian artery.

Blood Supply

The arterial supply to the reproductive system of the cow is provided by the ovarian, umbilical, vaginal, and internal pudendal arteries with their associated branches (Figure 22.11). The ovarian artery leaves the abdominal aorta, passing within the mesovarium to perfuse the ovary. The ovarian artery gives off branches to the uterine tube and the uterine horn [4]. The umbilical artery arises from the internal iliac artery near the pelvic inlet and sends out a uterine branch that supplies the cervix, body, and horns of the uterus. The vaginal artery arises directly from the internal iliac artery. The vaginal artery gives off a uterine branch that anastomoses with the uterine branch of the umbilical artery. The uterine artery formed by this anastomosis in turn anastomoses with the uterine branch of the ovarian artery, completing an arterial loop that supplies all the internal components of the reproductive system [4]. The internal pudendal artery is a direct continuation of the internal iliac artery. It courses caudally after the vaginal artery branches off within the ischiorectal fossa. The internal pudendal artery terminates as ventral perineal and dorsal labial branches [4].

The innervation of the external genitalia of the cow consists of the pudendal nerve and its branches. The pudendal nerve carries motor, sensory, and parasympathetic nerve fibers [2]. The pudendal nerve passes through the pelvic cavity medial to the sacrosciatic ligament and divides as it approaches the lesser ischiatic notch of the pelvis into proximal and distal cutaneous branches that supply the skin of the caudal hip and thigh [2, 3]. The pudendal nerve continues through the ischiorectal fossa and terminates as the dorsal nerve of the clitoris and a mammary branch [3]. Parasympathetic components of the pudendal nerve come by way of the pelvic nerve, which originates as a coalescence of branches of the sacral spinal nerves at the sacral plexus [4]. Sympathetic components of the pudendal nerve arise from the paired hypogastric nerves, which contribute sympathetic fibers from the caudal mesenteric plexus to the genital system [4].

Placenta

The cotyledonary bovine placenta is composed of the fetal cotyledons and the maternal caruncles that fuse and form the placentomes [4]. The placentomes are the sites for the transfer of maternal nutrients to the fetal circulation. The fetal membranes consist of two fluid-filled sacs. The innermost sac is the amnion, which surrounds the developing embryo; the outermost sac is the chorioallantois, which encircles the amnion and is composed of two separate tissues that fuse as the embryo develops. The outer layer is the chorion that develops from the trophoblast layer, which is the outer layer of the blastocyst of the embryo. As the embryo develops, the allantois, a sac that arises from the embryo hindgut, expands and eventually fuses with the chorion to form the chorioallantois.

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23

Initiation of Puberty in Heifers

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Introduction

Puberty is a critical physiological milestone in a heifer's reproductive life. In general terms, puberty can be defined as the process whereby animals become capable of reproducing themselves [1]. At the onset of puberty, a rise in plasma progesterone concentrations indicates cyclic ovarian activity before the first observed estrus [2]. Thus, puberty can be defined as the first day that serum progesterone (determined in blood samples collected at weekly intervals) exceeds 1 ng/ml [3]. Other authors define puberty as when ovulation is accompanied by visual signs of estrus with subsequent normal luteal function as evidenced by serum progesterone concentration above baseline for over two consecutive weeks [4]. Puberty involves a complex series of interactions between genetic, nutritional, and environmental factors that direct endocrine events that culminate in the attainment of reproductive capacity. In heifers, puberty is triggered when the hypothalamic–pituitary–gonadal axis first loses its sensitivity to the negative feedback effects of estradiol-17β, allowing a surge of luteinizing hormone (LH) to occur [5]. It is now accepted that puberty and first ovulation are not necessarily coincident since in most heifers "silent" ovulations and short luteal phases may occur during the peripubertal phase [5].

Endocrine Events

Puberty encompasses the transition from the anovular state to one of regular recurring ovulations. The mechanism by which the hypothalamic–pituitary–gonadal axis loses its sensitivity to the negative feedback effects of estradiol-17β has been the subject of research efforts for many years. The classical "gonadostat" theory, originally developed in a rodent model, appears applicable to cattle [6]. Ramirez et al. proposed that first ovulation results when sensitivity to steroid negative feedback diminishes, allowing sufficient gonadotropin output to drive follicular maturation, ovulation, and formation of a corpus luteum [6]. The hypothalamic–pituitary–gonadal axis of female cattle goes through several changes during its development. In utero, the fetus secretes gonadotropins for the first seven months of gestation. After this period, circulating gonadotropins are substantially reduced due to stimulation of the fetal central nervous system (CNS) [7]. In sheep, it has been demonstrated that the CNS-stimulated reduction in gonadotropin release that occurs in late gestation is mediated through inhibition of *N*-methyl-D-aspartate (NMDA) receptors, which have been demonstrated to be stimulatory to the gonadotropin-releasing hormone (GnRH) pulse generator nucleus in the fetal hypothalamus [8]. It has become well established that kisspeptin-1 (kiss-1) neurons are the final common pathway regulating GnRH neurons [9]. However, NMDA may also act directly on GnRH cell bodies in a kisspeptin-independent fashion [10]. Postnatally, mean plasma LH concentrations reach a maximum around three months of age, then slowly decline, before rising again and culminating in ovulation – typically around 10–11 months of age [11]. This transient increase in the circulating concentration of LH is associated with early follicular development and is thought to play a role in the timing of puberty.

In an attempt to hasten the onset of sexual maturity, Madgwick et al. [12] noted that the early rise in LH concentration was advanced by injecting heifer calves with GnRH twice daily from four to eight weeks of age. Treatment with GnRH increased mean circulating concentrations of LH at eight weeks of age, increased LH pulse frequency at four and eight weeks of age, and reduced the mean age at puberty by six weeks. Body weight gain was greater in

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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GnRH-treated calves than in control calves and the rate of weight gain was shown to be a significant covariate with age at puberty. This early transient rise in circulating LH stimulates ovarian follicular development, resulting in estradiol-17β synthesis that has a negative feedback effect on gonadotropin secretion [13].

Although an increase in circulating estradiol-17β has not been consistently demonstrated during this time period [14, 15], it is assumed the decline in LH is due to increased sensitivity to negative feedback by estradiol-17β on the hypothalamus–pituitary [14, 15]. From this point until just prior to puberty, estradiol-17β continues to exert negative feedback, after which sensitivity to the negative feedback effect of estradiol-17β gradually declines. This period is known as the peripubertal period and begins about 50 days before puberty [13, 16]. The decline in sensitivity to negative feedback by estradiol-17β has been associated with a reduction in the number of cytosolic estradiol-17β receptors in the anterior and medial-basal hypothalamus [16]. The result is that estradiol-17β becomes ineffective at suppressing LH secretion and an ovulatory surge of LH is released [17]. Progesterone levels are very low (300 pg/ml) in the peripubertal period, but there are two distinct elevations of progesterone prior to the first preovulatory peak of LH [18]. The return to baseline levels subsequent to the first elevation in progesterone is always followed by the priming peak of LH, while the second elevation in progesterone precedes the pubertal peak of LH [18]. The profile of concentrations of LH between the two major LH peaks, coincident with the second progesterone elevation, appears as a transition between prepubertal and postpubertal LH baseline concentrations. This suggests that progesterone plays a key role in the changes leading to the establishment of the phasic LH release characteristic of the postpubertal heifer by priming the hypothalamus to respond to E2 positive feedback [18]. During the peripuberal period, growth-related cues are monitored and regulate the activity of the GnRH pulse generator. When sufficient body size/composition is attained, the frequency of LH pulses increases because sensitivity to estradiol-17β inhibitory feedback decreases [14]. The high-frequency LH pulses stimulate follicular maturation and estradiol-17β accelerates the GnRH pulse generator, resulting in the ovulatory surge of LH [19].

Pro-opiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) comprise a critical metabolic-sensing pathway controlling the reproductive neuroendocrine axis. The POMC–kisspeptin pathway may be important in mediating the nutritional acceleration of puberty in heifers [20].

The first ovulation is not synonymous with puberty and the first luteal phase is typically of shorter than normal duration. Prostaglandin ($PGF_{2\alpha}$) released from the endometrium is responsible for the reduction in luteal lifespan (premature luteolysis) following first ovulation in heifers [21, 22]. Presumably, this occurs because of an abundance of endometrial oxytocin receptors that mediate release of $PGF_{2\alpha}$ [23]. Subsequently, endometrial oxytocin receptor concentration is downregulated by exposure to progesterone for 12–14 days [24]. Frequency of LH pulses increases during the 50 days preceding first ovulation and reach about one per hour around the time of first ovulation [17]. Amplitude of LH pulses also increases during this time, but increased pulse frequency appears to be critical for initial ovulation [17]. Follicle-stimulating hormone (FSH) concentrations in blood do not fluctuate as much as LH in the peripubertal period, suggesting that FSH may play more of a permissive role in the initiation of puberty [18]. Other hormones undoubtedly play a role in initiation of puberty, but LH appears to be of paramount significance. Prolactin may play a role in heifer puberty, but blood concentrations do not change at puberty as they do in bulls. There is abundant evidence to suggest that heifers are actually capable of ovulating from early on in life but fail to do so because of insufficient gonadotropic stimulation. In fact, McLeod et al. [25] were able to induce preovulatory gonadotropin surges in GnRH-treated five-month-old heifers. Even more impressive was the research of Seidel et al. [26] who induced ovulations in one-month-old heifers with gonadotropin administration. Although ovulations can be artificially induced with gonadotropins, it is notable that the hypothalamus–pituitary becomes increasingly sensitive to GnRH stimulation as the time of puberty approaches. It has been shown that the positive feedback effect of estradiol-17β on surge LH release becomes functional between three and five months of age [27]. Collectively, available data indicate heifers are capable of ovulating long before puberty but fail to do so spontaneously until the inhibitory effect of estradion-17β on GnRH release wanes.

Development of the Female Reproductive Tract

Overall, body growth and development of the reproductive tract occur in an asynchronous pattern. For example, the ovaries grow at a rate 2.7 times faster than the body until puberty, whereas the tubular reproductive tract grows at about the same rate as body growth until about six months of age, and then enters a period of accelerated development until puberty [28]. No ovarian follicles are macroscopically visible at birth, but their numbers increase to maximal at four months, decrease through eight months of age, and remain relatively constant thereafter [28]. Growing and antral follicles increase in number during the first three to four months of age, which corresponds to the transient

increase in circulating LH concentrations observed during that time period [29].

Height of the luminal epithelium of the tubular reproductive tract is stimulated at birth but regresses by one or two months of age. Thereafter, increases in height of epithelia are most rapid after six months. From this information it is concluded, at least for Holstein heifers, that rapid peripubertal growth of the reproductive tract commences during the seventh month and is largely terminated by 10 months of age [28].

Factors that Influence Age of Puberty

It is generally accepted that puberty in cattle occurs at around 9 or 10 months of age. However, there are reports of puberty occurring any time between 6 and 24 months [1], with anecdotal reports of heifers calving at 13 months indicating that puberty can occur as early as 4 months of age. Recently, the author assisted an induced delivery of a near-term calf in a 14-month-old Shorthorn heifer, indicating that puberty in this heifer must have been around 5 months of age. Age at puberty is influenced by body weight and composition, breed, nutrition, genetics, and season of the year. Any adverse factor that decreases prepubertal growth, such as protein-energy malnutrition, scours, pneumonia, parasitism, or harsh weather conditions, results in delay of the onset of puberty.

Weight and Body Composition

An early study by Sorensen [30] showed that attainment of puberty in heifers was more influenced by weight than age. He found that heifers on a higher plane of nutrition reached puberty at an earlier age than similar heifers with lower average daily gain [30]. However, heifers may have similar body weights but vary in frame size, indicating they have differing body composition. For any given frame size, heifers that are heavier reach puberty at an earlier age. Body weight at puberty has a heritability coefficient of 0.40 [31]. Overall, heifers reared on higher planes of nutrition are heavier but younger than nutritionally restricted animals at puberty [32]. However, puberty does not occur at similar body composition or metabolic status in all heifers [33] but is positively correlated with body fat percentage and negatively correlated with carcass moisture percentage [33]. The precise mechanisms involved in the relationship of body composition and puberty are not clearly defined. However, it is known that somatotropin and the insulin-like growth factor (IGF)-I system are involved [34]. This fact was demonstrated by Armstrong et al. [35] who immunized heifers at three or six months of age against growth hormone releasing factor. By 14 months of age 75% of control heifers but only 22% of immunized heifers had reached puberty and had significantly fewer follicles >7 mm by six months of age.

Breed

Age and weight at puberty are affected by several factors, including breed. Generally, breeds of a larger size at maturity are older and heavier upon reaching puberty [36]. A classic example of the effect of breed on puberty is illustrated in the study by Laster et al. [37]. Their group found that female progeny of a Charolais bull were 50 days older and 120 kg heavier at puberty than progeny of a Jersey bull when all dams were Angus cows. Although the Charolais × Angus heifers grew faster than the Jersey × Angus heifers, they did not reach puberty at as young an age as the Jersey × Angus heifers due to breed effect, which, in this case, overrode the influence of rate of gain. Generally, European breeds reach puberty younger but at slightly heavier weights than Hereford or Angus heifers [37]. In a study on the effects of heterosis on age at puberty, Wiltbank et al. [38] found half to three-fourths of the heterosis effect on age at puberty was independent of heterosis effects on average daily gain. Individual sire within a specific breed also has a significant effect on age of puberty of female offspring. The heritability coefficient for age at puberty is 0.41 [31].

Plane of Nutrition

Body energy reserves and metabolic state are relevant modifiers of puberty onset and fertility. Heifers in a peripubertal state may be induced to ovulate by abruptly increasing the plane of nutrition [39, 40]. A study by Chelikani et al. [41] involving Holstein heifers illustrates this point. Heifers were fed to gain 1.1, 0.8, or 0.5 kg/day from 100 kg liveweight. Age at puberty for the three groups was 9, 11, and 16 months, respectively. Precocious puberty (<300 days of age) can be induced in beef heifers by early weaning and feeding a high-concentrate diet [42]. The mechanism by which early weaning and feeding a high-concentrate diet results in precocious puberty in heifers is associated with advancement of the reduction of estradiol negative feedback on secretion of LH. The mechanisms linking nutrition and metabolism to puberty are not clearly understood, but it appears several neuropeptides operating in a reciprocal manner (orexigenic and anorexigenic) are involved.

Kisspeptin is a primary candidate for modulating the effects of nutrition on reproduction. Kisspeptin acts in the role as a final common pathway to integrate stimulatory and inhibitory inputs to influence GnRH synthesis and release. Much of the work with this hormone has

been in a rodent model, but one may speculate that the same mechanisms are operational in cattle. Kisspeptin signaling in the hypothalamus has been suggested as the primary positive regulator of the GnRH pulse generator [43]. In the mid-1990s, the adipose hormone leptin was shown to be an essential signal for transmitting metabolic information to the centers governing puberty and reproduction and kisspeptins have emerged as conduits for the metabolic regulation of reproduction and putative effectors of leptin actions on GnRH neurons [44]. Leptin is produced by adipocytes and regulated by long-term and recent nutritional status [45]. Circulating leptin concentrations increase as puberty approaches but do not change appreciably in response to dietary change when percentage of total carcass body fat is above a minimum threshold, indicating that a certain minimum body condition is required for puberty [46]. Ghrelin and other metabolic effector hormones known to modulate the hypothalamic–pituitary–gonadal axis, such as neuropeptide Y, melanocortins, and melanocyte-concentrating hormone, are additional putative regulators of the hypothalamic/kisspeptin system [44].

Genetic Markers for Age at Puberty

Two of the major factors that influence reproductive efficiency and hence profitability due to greater lifetime production in cattle are age at puberty and postpartum anestrous interval. Heifers that have an earlier age at puberty generally have greater lifetime productivity. Age at puberty is moderately heritable, so selection over multiple generations should reduce the number of days to puberty. We now have the technology to evaluate genetic markers for age at puberty in cattle. For example, quantitative trait loci have been identified that predict male reproductive traits including age at puberty in cattle [47]. Similarly, random amplified polymorphic DNA markers have been used for identifying Nelore bulls with early (precocious) or late (non-precocious) puberty [48]. In heifers, an association weight matrix (AWM) has been constructed based on 22 related traits with single nucleotide polymorphisms [49]. The AWM results recapitulated the known biology of puberty, captured experimentally using validated binding sites, and identified candidate genes and gene–gene interactions for further investigation. Takada et al. [50] evaluated Nelore heifers to detect known polymorphisms in candidate genes related to sexual precocity and identified five genes that influence sexual precocity. Advances in genomic technologies will likely provide a powerful tool for selecting heifers at birth that will have a greater probability of being reproductively successful if managed correctly [51].

Correlation Between Sire Scrotal Circumference and Age at Puberty of Daughters

Early studies reported a favorable correlation between yearling scrotal circumference (SC) in bulls and age at puberty in half-sib heifers in populations of purebred animals [52, 53]. Estimates of genetic correlation between SC in yearling bulls and age at puberty in half-sib heifers of −0.71 and −1.07 (favorable) have been reported by Brinks et al. [52] and King et al. [53] In a study designed to estimate genetic correlations between testicular measurements and female reproductive traits in Hereford cattle, the authors concluded that heritability of female reproductive traits tended to be low to moderate but "selection for increased testicular size would lead to improvement in female reproduction, particularly an increase in calving rate and a decrease in age at first breeding" [54]. A favorable relationship between SC in Brahman bulls and age at puberty in Brahman heifers reared under subtropical conditions has also been reported [55]. However, more recent work suggests that the correlation between genetic response in female reproductive traits (including age at puberty) and sire yearling SC may be expected to be less than previously reported in the literature. The study by Martinez-Velazquez et al. [56] calculated the correlation between yearling bull SC and age at puberty in daughters to be only −0.15. Morris et al. [57], in a 14-year study involving Angus cattle, reported a correlation of −0.25 between sire SC and age of puberty in heifer offspring. In an Australian study, no significant relationship was found between age at puberty in heifers and age and SC at puberty in related bulls [58]. Results of a subsequent experiment designed to investigate this correlation using Limousin bulls bred to crossbred cows indicated that selection of resulting replacement heifers based on sire SC phenotype did not significantly influence heifer age at puberty [59]. However, when sire SC estimated progeny difference was used instead, selection resulted in a significant reduction in heifer age at puberty. The authors concluded that when sires are selected for high SC estimated progeny difference within a crossbreeding system, a large percentage of heifers should reach puberty early enough to calve at two years of age or younger. Nonetheless, it has been reported that age of puberty in bulls, based on attainment of a critical SC, is correlated with age at puberty in female offspring [60]. One explanation of the conflicting data may be that heterosis has a significant effect on the percentage of heifers reaching puberty by 368, 410, and 452 days, with the greatest effect at the younger age [61].

Management Options to Influence Age at Puberty

Management of replacement beef heifers should focus on factors that enhance physiological processes that promote puberty [62]. Procedures that ultimately affect lifetime productivity and reproductive performance of heifers begin before birth and include decisions that involve growth-promoting implants, feeding, breed selection, calving and weaning date, social interaction, sire selection, and exogenous hormonal treatments to synchronize or induce estrus. This is especially relevant in systems where heifers are expected to calve by two years of age or where the breeding period is restricted [36]. Heifers that calve as two year olds produce more calves in their lifetime than heifers that calve as three year olds [63]. Breed and postweaning rate of gain have a large influence over onset of puberty [36]. For optimal fertility, heifers should not be bred at their pubertal estrus as calving rate has been reported to be 21% less than for heifers bred at third estrus [64]. This implies that heifers should reach puberty within one to three months before the age at which they will be bred in order to mitigate the reduced fertility associated with breeding at pubertal estrus.

Photoperiod

Consideration should be given to time of year when calving occurs. Although cattle are not considered to be strictly seasonal breeders, there is a seasonal effect on reproduction. For example, it has been reported that fertility, cycle length, and postpartum anestrous period length vary with season. More specifically, a winter environment (northern hemisphere) delays onset of puberty. Schillo et al. [65] reported that fall-born heifers were younger at puberty than those born in spring, and that heifers exposed to simulated changes in daylength from spring to fall after six months of age showed advanced onset of cyclic ovarian activity. The effect of season is likely mediated through photoperiod and is not a direct consequence of improved nutrient availability. As further evidence of this effect, puberty can be advanced in heifers by administration of melatonin-containing implants [66]. Treatment of three- to four-month-old winter-born heifers with exogenous melatonin for a period of five weeks at the beginning of summer significantly increased the incidence of animals attaining puberty by March of the following year (58 vs. 17%) [66]. Exposure of prepubertal heifers from 22 or 24 weeks of age until first ovulation to an artificially extended photoperiod will also advance first ovulation [67].

Growth-Promoting Implants

Growth-promoting implants are widely used in the beef industry. However, in heifers to be retained for breeding, implants containing estradiol, zernol, or trenbolone acetate inhibit the development of a mature reproductive endocrine system when administered to suckling beef heifers and consequently delay onset of puberty. For example, zeranol implants at birth delay the onset of puberty and decrease uterine horn diameter. Furthermore, zeranol-implanted heifers have lower pregnancy rates and higher rates of abortion than non-implanted herdmates [68]. In contrast, Rosasco et al. [69], in a study comparing performance of crossbred Angus heifers implanted with Synovex® C (Zoetis, Parsippany, NJ, USA) at three months of age, reported no deleterious effects on reproductive performance through four calving seasons (note: product label specifies for heifers 45 days and older up to 400 pounds). The implanted calves were heavier at weaning than control heifers such that producers who do not make selection of replacement heifers until weaning may expect some profit advantage from heifers not retained as replacements. Replacement heifers that are identified early in life should not be implanted as there is no benefit in terms of age at puberty or incidence of dystocia.

Use of Progestins to Advance Onset of Puberty

It is well established that progesterone sensitizes the hypothalamus to the positive feedback effect of estradiol-17β that results in the preovulatory LH surge. Progestin administration hastens puberty by accelerating the peripubertal decrease in estradiol-17β negative feedback effect, thereby facilitating the LH surge [72]. Furthermore, progesterone priming has been shown to enhance follicular estradiol-17β synthesis in ewes [70] and it has also been demonstrated that circulating estradiol-17β concentrations were higher at the first ovulatory estrus than in the preceding silent estrus [71]. As an example of the use of an exogenous progestin, melengestrol acetate (MGA) fed for eight days followed by withdrawal, in turn, enhanced onset of puberty by stimulating pulsatile LH secretion that accelerated follicle growth to the preovulatory stage [73]. In synchronization programs for heifers that may be peripubertal and prone to premature luteolysis, the incorporation of a progestin improves pregnancy rates. Treatment with some progestins, but not others, before first ovulation was able to eliminate the occurrence of short-lived corpora lutea [74–76]. Specifically, progesterone administered via intravaginal implant for five days was found to be more effective in

reducing incidence of premature luteolysis after GnRH-induced ovulation than oral MGA [75].

Nutritional Optimization of Puberty

Rapid growth from birth to weaning (6–10 months) and from weaning to puberty (11–16 months) is critical for beef heifer calves to attain puberty at an early age. It is now clear that the interval between four and nine months of age is a critical period for metabolically programming the neuroendocrine axis to achieve early reproductive maturation. The requirement for rapid growth in breeding heifers is to ensure that they will reach puberty in advance of the breeding season and be bred to calve by 24 months of age. Therefore heifers must exceed both the age and weight "threshold" to attain puberty. Nutritional programs for breeding heifers are designed to grow animals at a rate that allows them to exceed the weight threshold before or soon after the age threshold for puberty is surpassed [77]. Across several breeds, heifers were 55–60% of mature body weight at puberty [78]. The practice of developing heifers to reach a target body weight (typically ≥65% of mature weight) prior to breeding is commonplace in the industry. However, one must bear in mind that the target body weight will vary by breed. Methods to estimate effects of heifer nutrition protocols have been developed. For example, residual feed intake (RFI) is the residual from a regression model regressing feed intake on average daily gain (ADG) and body weight $(BW)^{-0.75}$ [79]. Body fat stores are greater in heifers with greater RFI than in their more efficient herdmates. A 1-unit increase in RFI resulted in a reduction of 7.54 days in age at puberty in *Bos taurus* beef heifers. However, *Bos indicus*-influenced heifers, which reach puberty at older ages, were not found to have sexual maturity influenced by selection for higher RFI [79]. From a management standpoint, selection for low RFI results in selection of leaner heifers that reach puberty at older ages [79]. Therefore, at some point, it becomes counterproductive to select replacement heifers on the basis of low RFI. This is because reproduction has been reported to be five times more important to commercial cattle producers than growth rate or milk production [80], and heifers that calve early in their first calving season tend to calve early throughout their lives and have greater lifetime calf production [81].

Precocious puberty (<300 days of age) in beef heifers can be induced by early weaning and continuous feeding of a high-concentrate diet. As in the case of progestin administration, puberty is preceded by increasing frequency of LH pulses [82]. Heifers experiencing induced precocious puberty weigh significantly less at puberty than their traditionally weaned and fed counterparts [82]. Furthermore, it has been determined that feeding a high-concentrate diet from 126 days (after weaning at 112 days) through 196 days was as effective at inducing precocious puberty as continuous high-concentrate feeding [83]. Taken together, data indicate that high preweaning growth rate and heavy weaning weights are associated with early puberty and heavier weight at puberty [84].

The concept of fetal programming was proposed by Barker [85] to explain a geographical association between poor maternal physique and health, poor fetal growth, and high death rates from cardiovascular disease in adult humans. Similarly, there are numerous studies in animals that demonstrate that transient adverse events in prenatal or early postnatal life have enduring and profound effects on physiology, although such effects may remain latent until the animal is mature. One example of this comes from experiments in which the nutrition of pregnant and lactating rats was manipulated. The adult body size of the offspring was more powerfully determined by their mothers' nutritional status during pregnancy and lactation than by their genetic constitution [86]. Prenatally undernourished female rats displayed delayed puberty which was associated with decreased hypothalamic kisspeptin action [87]. It is now established that maternal nutrient status can cause epigenetic alterations to the genome of the developing fetus, which can potentially impact future generations. Epigenetics is defined as heritable changes in gene expression resulting from alterations in chromatin structure but not in DNA sequence. Epigenetic programming can have lasting effects on future generations through intergenerational influences. These are described as factors, conditions, exposures, and environments in one generation that impact the health, growth, and development of subsequent generations. Three main mechanisms cause epigenetic changes to the genome: DNA methylation, histone modification, and non-coding microRNAs [88]. Non-coding RNAs are RNA transcripts that are not transcribed into proteins, but have been shown to regulate transcription, stability, or translation of protein-coding genes [89]. These processes regulate both the intensity and timing of gene expression during cell differentiation [90, 91]. In cattle, nutrient restriction of gestating cows resulted in heifer offspring with reduced wet ovarian weight and decreased luteal tissue mass compared with heifers born to control-fed cows [92]. Funston et al. [93] also reported that heifers born to cows protein-supplemented during the last one-third of pregnancy attained puberty 14 days earlier than heifers from non-supplemented dams. In sheep, fetal growth restriction altered pituitary LHβ expression and number of follicles in the fetal ovary [94]. Similarly in cattle, nutrient restriction for the first 110 days of gestation resulted in calves with fewer antral follicles compared with calves born to non-restricted cows [95]. This may impact the onset of puberty in heifers as there is evidence from ultrasonographic studies that development of ovarian

antral follicles and tubular genitalia occur in parallel [95] and this development is necessary for puberty.

In dairy heifers it appears that factors including colostrum intake, preweaning growth rate, and body composition influence age at puberty. As an example of the effect of preweaning (0–42 days) growth rate, heifers fed an intensive milk replacer diet were 15 days younger at first pregnancy and 14 days younger at calving than heifers fed a conventional milk replacer diet [96]. The conventional diet consisted of a standard milk replacer (21.5% crude protein [CP], 21.5% fat) fed at 1.2% of BW on a dry matter basis and starter grain (19.9% CP) to attain 0.45 kg of daily gain. The intensive diet consisted of a high-protein milk replacer (30.6% CP, 16.1% fat) fed at 2.1% of BW on a dry matter basis and starter grain (24.3% CP) to achieve 0.68 kg of daily gain [96].

Finally, one should bear in mind that consumption of certain feedstuffs may actually be deleterious to attainment of puberty. One such example is endophyte-infected tall fescue. Cattle consuming this forage are prone to decreased calving and growth rates, delayed onset of puberty, and impaired function of corpora lutea [97].

Effect of Heifer Temperament on Age at Puberty

A study by Cooke et al. [98] evaluated the influence of temperament on various performance measures including age at puberty in *Bos indicus* heifers. *Bos indicus* heifers classified as "excitable" (based on chute exit velocity) had reduced growth, increased plasma cortisol concentrations, and hindered puberty attainment compared to heifers classified as "adequate" or less excitable temperament.

Influence of Bull Exposure on Age at Puberty

Unlike other domestic species (sheep, goats, swine), exposure to a bull has no effect on the incidence of precocious puberty [99].

Influence of Age of Dam on Follicular Reserves

Walsh et al. [100] reported that maternal age affected the number of antral follicles detectable by ultrasonography in the ovaries of the daughters at a year of age. Holstein heifers that were born to heifers had fewer antral follicles detectable by ultrasonography at a year of age than Holstein heifers that were born to multiparous cows. Similarly, Angus heifers with diminished numbers of antral follicles detectable by ultrasonography (14.5 ± 0.8 follicles) had dams that were younger than the dams of Angus heifers with increased numbers of antral follicles (31.1 ± 0.8 follicles) [101]. These studies suggest that the lesser number of antral follicles detectable by ultrasonography in heifers born to primiparous dams is due to fewer ovarian follicle reserves. Selecting replacement heifers from mature dams may result in daughters with greater fertility and reproductive longevity; however, further research is necessary to determine if interactions between size of the ovarian follicle reserve and age at puberty influence fertility and reproductive longevity in replacement heifers.

Monitoring Heifers for Attainment of Puberty

The rearing of replacement heifers is a major financial investment for both beef and dairy cattle producers. The investment expenses do not begin to be recovered until after the first calf is weaned and sold, or in the case of dairy heifers, the onset of lactation, so having heifers calve at an optimal age is paramount to enterprise profitability. For this to occur it is essential that operators know when their heifers have attained puberty and become eligible for breeding. This is most critical for herds using a restricted breeding season.

Observation of signs of estrus can predict onset of puberty but is impractical for application to larger herds. Another observational tool, reproductive tract score (RTS), is a useful predictor of heifer fertility [102]. There is a positive correlation between high RTSs in heifers and percentage of heifers conceiving by artificial insemination [103]. RTS is a subjective estimate of sexual maturity based on ovarian follicular development and diameter of the uterine horns. An RTS of 1 is assigned to heifers with infantile tracts, as indicated by small, toneless uterine horns and small ovaries devoid of significant structures. Heifers with an RTS of 1 are likely the furthest from puberty at the time of examination. Heifers assigned an RTS of 2 are thought to be closer to puberty than those scoring 1, due primarily to larger uterine horns and ovaries. Those heifers assigned an RTS of 3 are thought to be on the verge of estrous cyclicity based on uterine tone and palpable follicles. Heifers assigned a score of 4 are considered to be cycling, as indicated by uterine tone and size, coiling of the uterine horns, and presence of a preovulatory-sized follicle. Heifers assigned an RTS of 4 do not have an easily distinguished corpus luteum. Heifers with an RTS of 5 are similar to those scoring 4 except for the presence of a palpable corpus luteum [104].

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Neuroendocrine Control of Estrus and Ovulation

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Introduction

The neuroendocrine control of the estrous cycle involves the integration of multiple regulatory signals. These signals form an intricate network that permits the fine control of gonadotropin release required for the complex sequence of ovarian follicular development, steroidogenesis, and ovulation. Secretion of gonadotropin-releasing hormone (GnRH) into the hypothalamic–adenohypophyseal portal circulation is considered the primary endocrinological mechanism regulating gonadotropin synthesis and release. Internal factors (e.g. gonadal and metabolic hormones, growth factors, and signaling molecules) and external factors (e.g. environmental chemicals, photoperiod, and stressors) are perceived at the level of the central nervous system and control GnRH secretion directly or through intermediate pathways. Under stimulation by GnRH, gonadotropes synthesize and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These two gonadotropins reach the gonads through the blood circulation and act in the ovary to stimulate follicle growth and maturation, ovulation of the preovulatory follicle, and synthesis of gonadal steroid and peptide hormones. Gonadal hormones, in turn, exert feedback actions at the central and adenohypophyseal levels to control the release of GnRH and gonadotropins. This intricate regulatory mechanism leads to dynamic changes in circulating concentrations of reproductive hormones during the estrous cycle (Figure 24.1).

The length of the bovine estrous cycle ranges from 17 to 24 days, averaging 21 days. Day 0 of the estrous cycle is considered the day on which the female exhibits estrus (standing to be mounted) and other proceptive behavior such as mounting herdmates, restlessness, and vocalization. Estrus is induced by an elevation in circulating concentrations of estradiol-17β associated with enhanced follicular steroidogenesis during follicle development [1]. Increased concentrations of estradiol reach a threshold 12–18 hours before onset of estrus, triggering a preovulatory surge of GnRH release and, consequently, a surge in gonadotropin release [2]. Onset of the preovulatory surge of LH generally occurs coincident with the onset of estrus. Ovulation is induced approximately 30 hours after the onset of estrus and is followed by an abrupt decline in circulating concentrations of estradiol. Following ovulation, follicular cells undergo functional transformation into luteal cells, which synthesize and release progesterone [1]. During corpus luteum formation and maturation, concentrations of progesterone in the circulation increase gradually to reach their peak approximately eight days after ovulation. The period of progesterone dominance is defined as the luteal phase of the estrous cycle. During the luteal phase, mean circulating concentrations of LH are relatively low due to infrequent episodic release of LH. In contrast, concentrations of FSH and estradiol fluctuate in association with waves of follicular growth and atresia [3, 4]. At approximately day 16–17 of the estrous cycle in the cow, luteolytic pulses of prostaglandin (PG)$F_{2\alpha}$ released by the endometrium cause luteal regression and a consequent decrease in circulating concentrations of progesterone [1]. Therefore, the progesterone restraint of GnRH/LH release that characterizes the luteal phase declines and the frequency of episodic release of GnRH/LH increases. Increased gonadotropin support promotes follicle growth and maturation, enhanced follicular steroidogenesis, elevations in the circulating concentrations of estradiol and, ultimately, estrous behavior. The period characterized by low circulating concentrations of progesterone and enhanced follicular development leading to ovulation is termed the follicular phase. The cyclic changes in

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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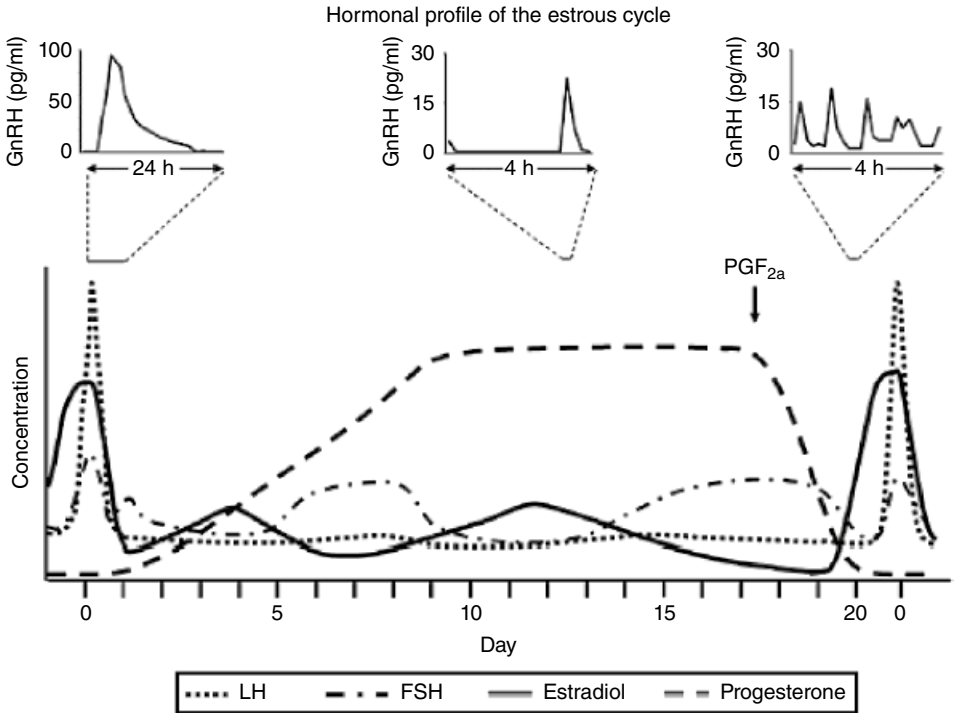


Figure 24.1 Relative changes in circulating concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and progesterone during the estrous cycle in cows. The day on which estrous behavior is observed is denoted as day 0 of the estrous cycle. Circulating concentrations of progesterone increase after ovulation with formation and maturation of the corpus luteum, and remain elevated for the duration of the luteal phase. Release of prostaglandin (PG)$F_{2\alpha}$ causes luteolysis, which is followed by a decrease in circulating concentrations of progesterone. The period of rapid follicular growth and maturation, and elevated concentrations of estradiol in circulation, that follows luteolysis characterizes the follicular phase. Elevated estradiol, in the absence of progesterone, leads to estrous behavior and the preovulatory surge of gonadotropin-releasing hormone (GnRH; top, graph on left). During the luteal phase, progesterone inhibits the frequency of pulsatile release of GnRH (top, graph in middle) and consequently the pulsatile release of LH. During the follicular phase, escape from progesterone inhibition leads to increased frequency of GnRH (top, graph on right) and LH pulses, which supports follicular maturation and enhanced ovarian steroidogenesis. Fluctuations in circulating concentrations of FSH and estradiol are observed in association with initiation and progression of follicular waves.

gonadotropin and ovarian hormone release, and resulting follicular activity, are usually recurrent, except when pregnancy is established or pathological conditions (e.g. undernutrition, disruption of normal luteolysis) are introduced.

The dynamic changes in concentrations of circulating LH and FSH during the estrous cycle are largely representative of distinct changes in the pattern of episodic secretion of GnRH. During the luteal phase, the frequency of episodic release of GnRH, and the correspondent pulses of LH [5], is approximately one pulse every four to six hours [2]. During the follicular phase, the episodic release of GnRH/LH is increased to approximately one pulse per hour [2, 5]. The follicular phase pattern of GnRH/LH release is essential for final follicle development and maturation, and enhanced steroidogenesis. The elevation in circulating concentrations of estradiol during late follicular phase leads to the preovulatory surge of GnRH. The GnRH surge is characterized by a sustained increase in concentrations of GnRH in the hypophyseal portal blood. At the onset of the surge, the pulsatile release of GnRH is of high frequency and high amplitude. With progression of the surge, the pulsatile pattern of GnRH release switches to a continuous elevation of GnRH in the portal blood [2]. This leads in turn to a massive discharge of LH from the adenohypophysis into the peripheral circulation, similar to that of GnRH in the portal circulation [2, 5]. Therefore two modes of endogenous GnRH/LH secretion are characteristic of the estrous cycle: the tonic episodic release and the preovulatory surge release [6]. These patterns of GnRH/LH release are regulated primarily by estradiol and progesterone, and their negative and positive feedback effects are mediated at the hypothalamic and adenohypophyseal levels.

Functional Anatomy of the Hypothalamic–Hypophyseal Unit

The cell bodies of neurons that synthesize GnRH in cattle are located in a continuum that extends from the preoptic area to the mediobasal hypothalamic region of the

diencephalon [7, 8]. GnRH neurons project to the median eminence, where GnRH is stored in secretory vesicles and released adjacent to capillaries of the hypothalamic–hypophyseal portal vasculature upon stimulation. Through this specialized vasculature that connects the hypothalamus and the adenohypophysis (Figure 24.2), GnRH reaches the adenohypophysis in functionally relevant concentrations, binds to the GnRH receptor on the surface of gonadotropes, and stimulates synthesis and release of gonadotropins. Disruption of the hypothalamic–hypophyseal vasculature by surgical lesion leads to decreased adenohypophyseal content and circulating concentrations of LH and FSH due to the lack of gonadotrope stimulation by GnRH [9, 10]. The transport of GnRH secreted from GnRH neurons in the median eminence is largely regulated by afferent neuronal signals and factors released by glial cells [11, 12]. Evidence for structural changes in glial cells that surround the capillaries of the median eminence also exists [13] and may contribute to the regulation of GnRH diffusion into the hypothalamic–hypophyseal circulation following a secretory episode.

Hierarchical Control of Gonadotropin Secretion: The GnRH Pulse Generator

In the early 2000s, the discovery and characterization of the hypothalamic neuropeptide kisspeptin, its functional roles in both pathological (hypogonadotropic hypogonadism) and physiological states (puberty), and ultimately its direct regulatory effects on secretion of GnRH revolutionized our understanding of how GnRH neurons are regulated [14–23]. Kisspeptin, a product of the *Kiss1* gene, is expressed in two discrete populations of neurons located in the preoptic/periventricular area and in the arcuate nucleus of the hypothalamus [24]. Kisspeptin is processed from a precursor peptide to produce several isoforms that vary in length. All biologically active forms of kisspeptin contain the C-terminus sequence common to all forms. The decapeptide form of kisspeptin appears to be the shortest isoform with full capacity for stimulation of GnRH secretion. The role of kisspeptin in the regulation of reproductive function was first characterized in studies investigating mutations in the kisspeptin receptor (*Kiss1r*) gene [14, 15]. Humans and mice with loss of function of kisspeptin receptors exhibit hypogonadotropic hypogonadism. Following these studies, the action of kisspeptin as a potent GnRH secretagogue was characterized in several species including cattle [19–23]. GnRH neurons contain the kisspeptin receptor, and kisspeptin acts directly on GnRH neurons at the level of the cell body and dendrites, as well as at the level of the terminals in the median eminence. Indirect effects of kisspeptin via modulation of other neuronal inputs to GnRH neurons [21] and direct action on adenohypophyseal cells *in vitro* [25] have also been reported. However, the physiological relevance for a direct action of kisspeptin in the adenohypophysis is unclear because kisspeptin fails to stimulate release of LH in hypothalamic–hypophyseal disconnected sheep [22].

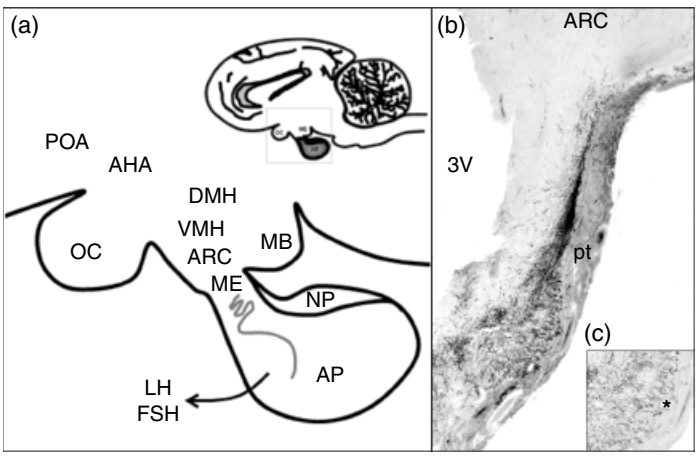


Figure 24.2 Sagittal view of the lower portion of the bovine brain and the hypothalamic areas involved in the neuroendocrine control of reproduction. In cattle, GnRH neuronal cell bodies are distributed in a continuum that extends from the septum/preoptic area (POA) to the basal portions of the hypothalamus (a). GnRH neurons project to the median eminence (ME) where neuronal terminals are adjacent to capillaries of the hypothalamic–hypophyseal portal system. GnRH released in the ME reaches gonadotropes in the adenohypophysis (AP) and stimulates synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Low (b) and high (c) magnification images of GnRH-immunoreactive neuronal fibers that project basolaterally toward the ME and terminate adjacent to capillaries (*) of the zona externa of the ME. AHA, Anterior hypothalamic area; ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; MB, mammillary body; NP, neurohypophysis; OC, optic chiasm; pt, pars tuberalis of the adenohypophysis; POA, preoptic area; VMH, ventromedial hypothalamus; 3V, third ventricle.

GnRH is the sole hypothalamic neuropeptide responsible for the secretion of pituitary gonadotropins [26]. It is released in a pulsatile pattern into hypophyseal portal vessels of the median eminence as a consequence of synchronous secretory activity of GnRH neurons located within the medial basal hypothalamus. This activity is characterized by volleys of multiunit electrical activity that can be detected by placing electrodes in the vicinity of these neurons [27–29]. A pulse of GnRH in hypophyseal portal blood precedes a corresponding pulse of LH released from the anterior pituitary into the peripheral circulation [30]. Occasionally, pulses of GnRH with very small amplitude do not result in a detectable LH pulse. During the estrous cycle, the high-frequency, relatively low-amplitude pattern of pulsatile LH release observed during the follicular phase is converted to a much lower frequency, high amplitude pattern during the luteal phase due to high circulating concentrations of progesterone [2]. When measured using highly specific radioimmunoassays, the episodic pattern of FSH secretion observed in the peripheral bloodstream is less distinct than for LH [31]. Although FSH secretion is driven ultimately by hypothalamic GnRH release, other factors secreted from both the dominant follicle (inhibin, activin, estradiol-17β) and locally in the pituitary (activin) significantly modulate changes in its peripheral plasma concentration, as discussed in the section "Differential Control of Gonadotropins: FSH".

The phenomenon of synchronous release of GnRH from hypothalamic GnRH neurons has been referred to historically as the GnRH pulse generator [32, 33]. Until recently, the mechanistic basis of the pulse generator has remained elusive. Based on *in vitro* cultures of immortalized GnRH neurons (GT1 cells), the idea that GnRH neurons have the intrinsic ability to generate autonomous pulses of GnRH was once widely accepted [34]. However, none of these studies demonstrated the ability of GnRH neurons to communicate with surrounding cohorts to stimulate a pattern of synchronous release. More recently, the concept of extrinsic pulse generation has gained favor with the discovery of specialized neurons that release the neuropeptide kisspeptin. The latter is released from two subsets of kisspeptin-expressing neurons, one located in the preoptic area and the other in the arcuate nucleus of the medial basal hypothalamus [35, 36]. Those within the arcuate nucleus localize three peptides, kisspeptin, neurokinin B (NKB), and dynorphin, and are now called KNDy neurons. KNDy neurons co-express receptors for NKB and dynorphin and secrete kisspeptin in response to their own release of NKB. This results in GnRH release from both GnRH cell bodies and their terminals in the median eminence [37, 38]. The activation of KNDy neurons results in release of dynorphin which inhibits KNDy neuron activity (Figure 24.3, left panels). This repeating cascade of KNDy neuron activity has provided convincing evidence that

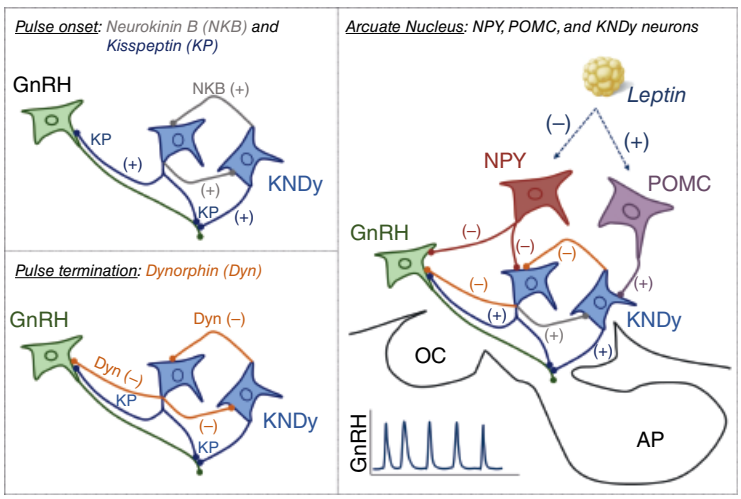


Figure 24.3 Proposed model for the neuroendocrine control of the pulsatile secretion of GnRH. *Top left panel*: Neurons co-expressing neurokinin B (NKB), kisspeptin (KP), and dynorphin (Dyn; termed KNDy neurons; blue) are a critical component of the GnRH pulse generator. Each GnRH pulse is initiated by NKB (gray) acting upon KNDy neurons (autocrine/paracrine) to stimulate kisspeptin (blue) release. Kisspeptin stimulates GnRH neurons (green) to depolarize and secrete GnRH in a pulsatile manner. *Bottom left panel*: GnRH release is terminated by the release of dynorphin (orange) from KNDy neurons acting directly on KNDy neurons (autocrine/ paracrine) and GnRH neurons. Dynorphin acts via the κ opioid receptor as an inhibitory neuropeptide, thus hyperpolarizing GnRH and KNDy neurons and terminating the GnRH pulses. *Right panel*: Leptin regulates episodic GnRH/LH secretion by acting upon an upstream hypothalamic neurocircuitry that includes neurons expressing neuropeptide Y (NPY; red) and pro-opiomelanocortin (POMC; purple). NPY inhibits GnRH secretion by acting directly on GnRH neurons as well as via KNDy neurons. POMC neurons stimulate GnRH secretion by acting primarily on KNDy neurons in the arcuate nucleus.

KNDy neurons serve as an extrinsic GnRH pulse generator [39–41]. In the ewe and in rodents, both NKB and dynorphin are absent from kisspeptin neurons in the rostral part of the hypothalamus [41]. Nonetheless, this population of cells does contain estrogen receptor alpha (ES1) [24]. Several studies have demonstrated that estradiol suppresses *Kiss1* expression in the arcuate nucleus while positively modulating kisspeptin neurons in the rostral hypothalamus [42, 43]. If ES1 is knocked out in all kisspeptin-expressing neurons, the onset of puberty can be advanced [42]. In ruminants, there is clear evidence supporting the concept that estradiol inherently restrains GnRH/LH secretion, ovarian function, and pubertal onset through ES1 [44–48]. Disturbance within the kisspeptin signaling network impedes secretion of GnRH, resulting in the failure to attain puberty [14, 15]. However, in conditions associated with inadequate kisspeptin synthesis, this deficit can be overcome by both peripheral and central administration of kisspeptin [18, 23].

Role of Gonadal Steroids

Positive and negative feedback actions of estradiol and progesterone at the level of the hypothalamus and adenohypophysis explain most of the alterations in the patterns of GnRH and LH release during the estrous cycle (Figure 24.4). Estradiol has a profound effect on the expression of the *Kiss1* gene, but estradiol actions differ between the two populations of kisspeptin neurons. In the preoptic/periventricular area, estradiol stimulates *Kiss1* gene expression. In contrast, estradiol inhibits *Kiss1* gene expression in the arcuate nucleus [49, 50]. This effect of estradiol on regulation of *Kiss1* expression may be involved in the feedback actions of estradiol on gonadotropin release. In ewes, estradiol induces activation of kisspeptin neurons in the preoptic/periventricular areas, indicating that this population of neurons may be important for mediating estradiol positive feedback and onset of the preovulatory surge of LH [51, 52]. Although controversial, the population of kisspeptin neurons in the

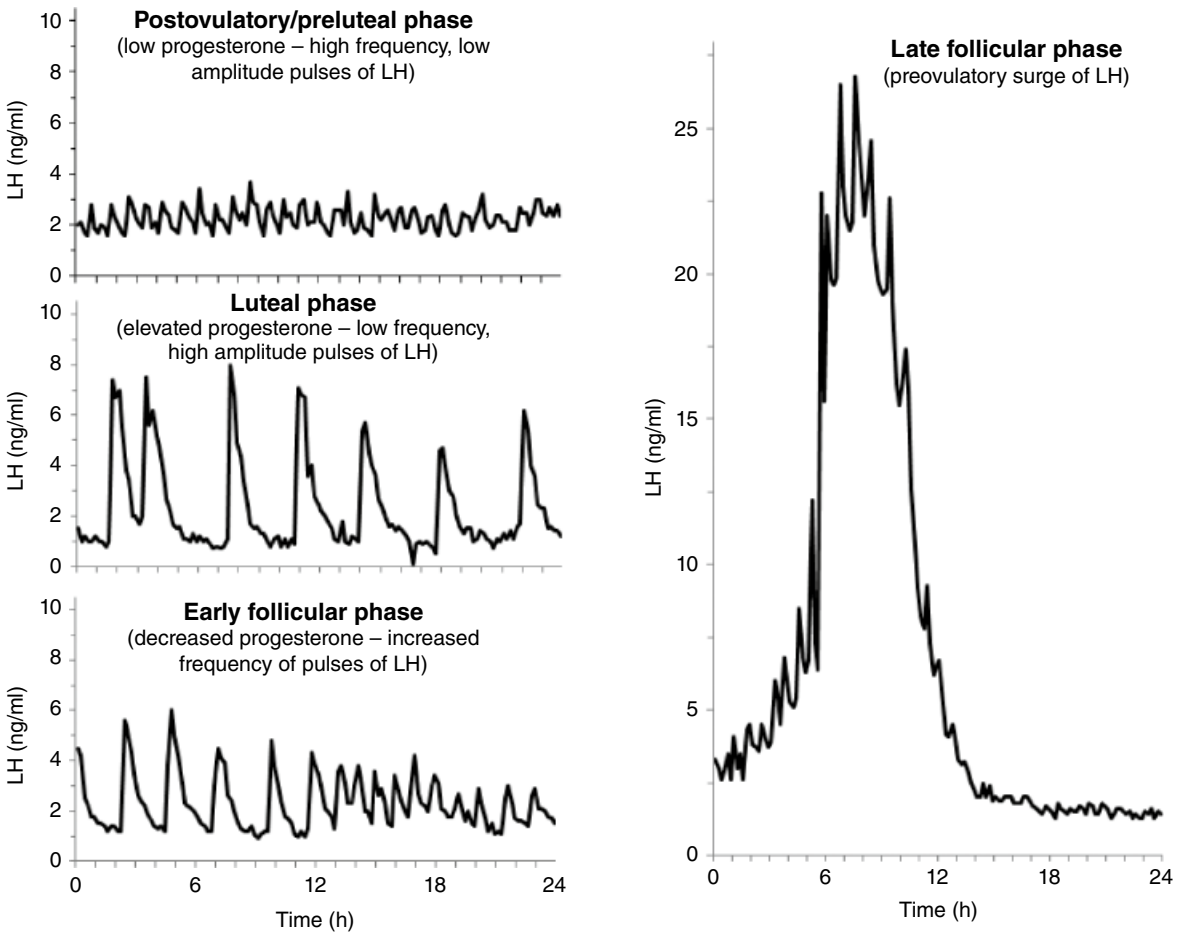


Figure 24.4 Patterns of release of luteinizing hormone (LH) during the estrous cycle in cows. Blood samples from the jugular vein were collected at 10-minute intervals for 24 hours in four distinct phases of the estrous cycle in a cow. *Source:* From [2], © 1980, Oxford University Press.

arcuate nucleus may also be activated during the preovulatory surge of GnRH/LH release. Nevertheless, the observation that constant exogenous treatment with kisspeptin induces sustained release of LH in anestrous ewes and synchronizes the LH surge [53, 54] has promoted investigations seeking to utilize kisspeptin, or its analogs, for pharmacological control of the estrous cycle in domestic livestock, including cattle.

The effect of progesterone in controlling the episodic release of GnRH/LH is well characterized: progesterone inhibits the frequency of LH release [55] by its action at the level of the hypothalamus [56, 57]. However, the negative feedback effect of estradiol is more complex and appears largely additive to the action of progesterone [58]. Estradiol positive feedback is the primary physiological mechanism driving the development of the preovulatory surge of GnRH and LH release during the late follicular phase [2, 5, 6]. Interestingly, although progesterone can block the estradiol-induced LH surge in heifers and ewes [59, 60], prior exposure to endogenous (luteal phase) or exogenous (pharmacological treatment) progesterone enhances the estradiol-induced LH surge [61].

The mechanisms by which progesterone inhibits the frequency of episodic release of LH appear to be mediated by endogenous opioid peptides, particularly dynorphin [62, 63]. Studies in sheep have indicated that progesterone, by acting on progesterone receptor-containing dynorphin neurons located in the arcuate nucleus of the hypothalamus, inhibits the frequency of GnRH pulses via activation of endogenous opioid peptide neurotransmission. This effect appears to be mediated by the κ opioid receptor within KNDy neurons. Support for this mechanism of action is demonstrated by the observations that (i) intracerebroventricular injection of progesterone inhibits the pulsatile release of LH [57]; (ii) antagonists of endogenous opioid peptide [64] and of the κ opioid receptor [63], injected in the mediobasal hypothalamus, increase the frequency of LH pulses; and (iii) progesterone receptor-containing dynorphin neurons, located in the arcuate nucleus of the hypothalamus and in KNDy neurons in particular, project extensively to GnRH neurons in the mediobasal hypothalamus, but to a lesser extent to GnRH neurons in the preoptic area [63]. Importantly, the population of GnRH neurons located in the mediobasal hypothalamus appears to be the major group of GnRH neurons activated during an LH pulse in sheep [65]. This provides compelling evidence that the neuronal circuitry located in this area represents a pathway by which progesterone inhibits the frequency of GnRH/LH episodic release during the luteal phase. Although the mechanisms of progesterone negative feedback on regulation of LH release have not been extensively investigated in cattle, evidence indicates that endogenous opioid peptides have a role similar to that characterized in sheep [66].

The estradiol negative feedback actions on LH release are more complex. During periods of elevated progesterone (e.g. luteal phase), estradiol enhances progesterone inhibition of LH pulsatility [57]. This effect may involve direct actions of estradiol on endogenous opioid peptide neurons, including arcuate nucleus dynorphin neurons that are known to express ESR1 and progesterone receptor in sheep [67, 68]. Because estradiol increases the expression of progesterone receptor in the arcuate nucleus of ovariectomized ewes [69], estradiol's enhancement of progesterone negative feedback may involve increased responsiveness of hypothalamic neurons to progesterone actions. Estradiol negative feedback is also demonstrated by the ability of estradiol to decrease the amplitude of LH pulses during the follicular phase in ewes [70]. However, this effect may be a consequence of the increased frequency of LH release observed during the late follicular phase [2] or by inhibition of gene expression of the α-subunit of glycoprotein hormones (common to LH, FSH, and thyrotropin) and the β-subunit of LH [71, 72].

Estradiol appears to have a biphasic effect on secretion of LH during progression of the follicular phase. Administration of estradiol at physiological doses capable of inducing the preovulatory surge of LH leads initially to an acute reduction in the frequency of LH release [70, 73]. Evidence for this effect also exists during the late follicular phase of the estrous cycle [5]. The brief period (approximately six hours) of estradiol-induced inhibitory tone is followed by a considerable increase in the frequency and amplitude of GnRH/LH pulses and, subsequently, progression toward a large sustained elevation in concentrations of LH characteristic of the preovulatory surge (estradiol positive feedback; Figure 24.5) [5, 6]. Although the mechanisms by which estradiol exerts the positive feedback are unclear, it appears to involve activation of estradiol-receptive neurons in the hypothalamus and transmission of the estradiol signal to GnRH neurons for stimulation of the surge release at the median eminence [74]. The ventromedial hypothalamus and the arcuate nucleus have been identified as critical sites for the positive effect of estradiol in ewes [75, 76]. Changes in norepinephrine, β-endorphin, glutamate and γ-aminobutyric acid (GABA), neurokinin B, and kisspeptin transmission may be implicated in the transmission of estradiol positive feedback. At the level of the adenohypophysis, estradiol enhances expression of the GnRH receptor [77]. This effect is physiologically relevant because an increased response of the gonadotrope to GnRH during the late follicular phase might be critical for maintaining elevated secretion of LH during the preovulatory GnRH surge.

Prior exposure to progesterone enhances the estradiol-induced surge of LH release. This effect of progesterone appears to occur at both hypothalamic and adenohypophyseal

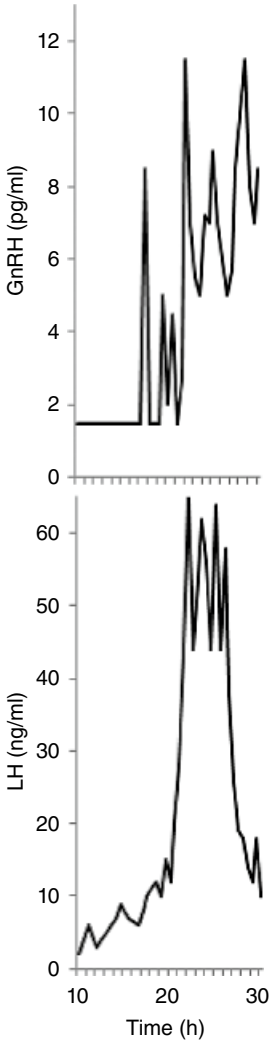


Figure 24.5 Estradiol-induced GnRH and LH surges in cows. Estradiol (1 mg) was injected intramuscularly at hour 0. Cerebrospinal fluid (CSF) from the third ventricle and blood from the jugular vein were obtained at 10-minute intervals. Concentrations of GnRH in the CSF (*top*) increased considerably and remained elevated for longer than 12 hours. Concentrations of LH (*bottom*) increased consistently with elevations in GnRH and returned to pre-surge concentrations in approximately 10 hours. *Source:* Modified from [5].

levels. In ovariectomized ewes, the magnitude of the GnRH surge was greater in ewes pretreated with progesterone during an artificial estrous cycle than in ewes not pre-exposed to progesterone [61]. Although evidence indicates that this effect occurs by alterations in GnRH secretion, direct effects in the adenohypophysis also occur. In ovariectomized ewes with the hypothalamic–adenohypophyseal connection surgically blocked, responsiveness to estradiol-induced surge of LH was enhanced in ewes pretreated with progesterone [78]. Progesterone priming appears to influence responsiveness to estradiol feedback in cows as well [79, 80].

Differential Control of Gonadotropins: FSH

The control of FSH synthesis and release from gonadotropes is exquisitely regulated by GnRH and gonadal hormones, including steroid and protein hormones. Intra-adenohypophyseal factors may also have a role in regulating FSH secretion by paracrine and autocrine actions [31]. In addition, the existence of a hypothalamic factor with FSH-releasing activity distinct from GnRH has been proposed [81], although such a factor is yet to be identified.

Similar to the release of LH, release of FSH has been shown to be pulsatile [82, 83]. However, using antisera produced against the β-subunit of FSH [84], which eliminates possible cross-reactivity with LH, yielded results in which distinct pulses of FSH release were difficult to detect in the peripheral circulation. This has been attributed in part to the long half-life of FSH in circulation [31]. In addition, fluctuations in concentrations of ovarian peptides (inhibin, follistatin) and estradiol that occur during the estrous cycle play major roles in regulating the pattern of secretion of FSH in cattle [3, 4, 85]. Nevertheless, studies in which concentrations of FSH were determined in blood samples collected from the hypophyseal portal vasculature in sheep have demonstrated that secretion of FSH is clearly pulsatile [31]. Interestingly, although a considerable proportion of the FSH pulses coincide with episodes of GnRH release, many non-GnRH-associated episodes are also observed.

The GnRH-associated pulses of FSH observed in sheep occur coincidently with pulses of LH. In sheep, LH and FSH are co-synthesized in a subpopulation of gonadotropes [86], although the two gonadotropins are stored in distinct vesicles. Therefore the coincident release of LH and FSH could reflect the concurrent exocytosis of readily releasable pools of vesicles containing gonadotropins. In cattle, however, there is a clearer distinction in FSH- and LH-synthesizing cells in the adenohypophysis [87]. Nevertheless, FSH is clearly secreted following GnRH administration in cattle [82, 88]. In addition, the preovulatory surge of LH is accompanied by a surge in FSH [89, 90]. These observations indicate that although FSH and LH might be synthesized in distinct populations of gonadotropes, both types of gonadotropes are responsive to GnRH. However, the control of secretion of each gonadotropin appears independent, and the interaction of GnRH-dependent and GnRH-independent mechanisms is important for the fine control of FSH secretion.

The mechanisms by which the non-GnRH-associated pulses of FSH are generated remain unclear. Direct actions of gonadal hormones at the level of the adenohypophysis are believed to be a major regulatory mechanism. Peptide (e.g. inhibin) and steroid (e.g. estradiol) hormones are

likely to control FSH synthesis and release at the level of the gonadotrope. Inhibin, a dimer of α and βA (inhibin A) or βB (inhibin B) subunits, is produced in follicular cells and acts as an endocrine factor regulating FSH release in gonadotropes. Inhibin suppresses FSH release in bovine pituitary cell cultures [91]. The role of inhibin has also been shown in immunization studies. Basal circulating concentration of FSH and GnRH-stimulated FSH release is increased in ewes immunized against bovine inhibin [92]. Similarly, mean concentrations of FSH increase in heifers immunized against inhibin, an effect associated with an increased number of small follicles present in the ovary [93]. The effect of inhibin on the regulation of gonadotropin release appears specific to the control of FSH release because there is no clear effect of inhibin on the release of LH [94]. In addition, it is unlikely that inhibin acts at the hypothalamic level for the control of GnRH secretion [95, 96].

Estradiol has been demonstrated to inhibit FSH synthesis and secretion in the adenohypophysis. The direct action of estradiol appears to occur through inhibition of *FSH-β* gene expression [72]. However, studies investigating the replacement of estradiol and inhibin in ovariectomized ewes indicate that a combination of estradiol and inhibin negative feedback is required for full inhibition of the post-ovariectomy rise in concentrations of FSH [97]. Additional ovarian hormones may contribute to the regulation of FSH release during the estrous cycle. Activin, a dimer of the inhibin β subunits (βAβA, βAβB, and βBβB), and follistatin, a β-subunit-binding protein, are produced in follicular cells and can regulate FSH secretion. Although the endocrine actions of ovarian-derived activin and follistatin are unclear, these proteins, as well as inhibin, are synthesized in the adenohypophysis and may control FSH synthesis and release by autocrine and paracrine mechanisms [98, 99]. Activin stimulates FSH synthesis and release, while follistatin inhibits activin actions by averting activin binding to the receptor. Inhibin, on the other hand, disrupts activin actions by binding to the activin receptor and inducing intracellular signaling via a β-glycan-mediated pathway. Therefore the postovulatory elevation in circulating concentrations of FSH observed shortly after the preovulatory LH and FSH surges (Figure 24.1) is largely explained by changes in feedback actions of gonadal hormones at the level of the adenohypophysis following disappearance of the dominant follicle after ovulation and decreased circulating concentrations of estradiol and inhibin. Increases in FSH in circulation at this time contribute to recruitment of the first follicular wave observed during the early luteal phase and at subsequent waves during the remainder of the cycle.

The existence of a distinct molecule of hypothalamic origin with the ability to regulate FSH release has been proposed. Evidence in support of this hypothesis includes the observations that ablation of anterior hypothalamic neurons and their projections impair release of FSH, without clear effects on release of LH [100, 101]. In addition, inhibition of LH release without notable effects on FSH release has been observed after immunization against GnRH and inhibition of GnRH actions by administration of GnRH antagonists in sheep [31, 102]. Nevertheless, isolation and identification of a molecule that can be characterized as an FSH-releasing factor distinct from GnRH would be required for confirmation of this hypothesis. Interestingly, lamprey GnRH III (lGnRH III) has been reported to selectively release FSH in cattle and was proposed as the mammalian ortholog for the mammalian FSH releasing hormone [103]. However, an in-depth evaluation of this hypothesis revealed that in fact lGnRH III acts as a weak competitor for the mammalian GnRH receptor and does not selectively release FSH in the bovine [88].

Nutritional Influences on the Hypothalamic–Hypophyseal Axis

The nutritional control of gonadotropin release occurs primarily through the regulation of GnRH release in the median eminence. Severe nutrient restriction suppresses secretion of LH in reproductively mature and prepubertal cattle [104, 105]. However, short-term restriction of feed intake has a differential impact on gonadotropin release in mature and immature female cattle. In cows, the frequency of episodes of LH release is not affected by two to three days of fasting [106, 107]. In contrast, fasting for 48–72 hours reduces LH pulsatility in prepubertal heifers [108, 109]. Gonadal steroids have a marked effect on the nutritional regulation of gonadotropin release. Pulsatile release of LH is suppressed in ovariectomized estradiol-treated rats fasted for 48 hours [110]. However, acute feed restriction does not suppress LH release in ovariectomized rats without estradiol replacement [110]. Thus, gonadal steroid hormones interact with nutritional and metabolic signals to regulate gonadotropin release.

Studies determining the concentrations of GnRH in blood samples collected from the hypothalamic–hypophyseal portal vasculature of normal-fed and feed-restricted ewes have demonstrated a considerable reduction in the frequency of GnRH pulses in response to feed restriction [111]. The reduction in the frequency of LH pulses was greater than the reduction in the episodic release of GnRH because some low-amplitude pulses of GnRH were not accompanied by a pulse of LH. Therefore, diminished amplitude of GnRH pulses may also play a role in the overall reduction in mean concentrations of LH observed during undernutrition. A major consequence of low frequency of GnRH stimulation

of gonadotropes in response to feed restriction is the decrease in synthesis of LH and GnRH receptors in the adenohypophysis [112]. Pulsatile administration of GnRH in feed-restricted ewes restores circulating concentrations of LH to normal and increases pituitary expression of *LH-β* and *CGA* (α-subunit) genes [113]. These observations are in concordance with the hypothesis that a high frequency of GnRH stimulation is necessary for optimal expression of the common α-subunit of adenohypophyseal glycoprotein hormones and the β-subunit of LH [114]. Therefore, in conditions of low synthesis and storage of gonadotropins in the adenohypophysis and diminished numbers of GnRH receptors in gonadotropes, small-amplitude pulses of GnRH may not be sufficient to stimulate a clear episode of LH release. This may explain the observation that some small-amplitude pulses of GnRH are not followed by an episode of LH release [111]. In addition, it is possible that small-amplitude pulses of GnRH prime the adenohypophysis for enhanced responsiveness to large-amplitude pulses that induce clear episodes of gonadotropin release [115].

Nutritional and metabolic status are perceived largely at the level of the hypothalamus, although integration with signals controlling other areas of the brain, as well as peripheral organs, are important for the precise control of gonadotropin secretion and action in target tissues. The signals that mediate the nutritional control of gonadotropin release include nutrients (e.g. glucose, fatty acids, amino acids) and metabolic hormones (e.g. leptin, insulin, ghrelin). Glucose serves as a major energy source for the central nervous system and, along with fatty acids and amino acids, serve as signaling molecules to control cellular metabolic pathways [116–118]. In sheep, insulin-induced hypoglycemia [119] and treatment with 2-deoxyglucose [120], a competitive antagonist of glucose metabolism, inhibit secretion of LH. In cattle, hypoglycemia induced by phlorizin, an inhibitor of glucose transporter that increases renal excretion of glucose, was observed to lead to similar decrease in concentrations of LH [121]. Although it is generally accepted that glucose is essential for normal hypothalamic function, the mechanisms by which glucose deficiency impairs the release of LH are unclear. Restoration of LH pulsatility in fasted monkeys occurs not only after feeding carbohydrate-based meals, which increase circulating glucose, but also after feeding protein-/fat-based meals, which do not elevate blood glucose [122]. Amino acids and fatty acids, in addition to serving as important precursor molecules for cellular biosynthesis, appear to also serve as signaling molecules for the control of reproductive neuroendocrine function [116]. Glutamate and the glutamate-derived GABA are neurotransmitters that act directly on GnRH neurons to regulate firing activity and GnRH release [123, 124].

Hormones such as insulin, insulin-like growth factor (IGF)-1, ghrelin, and leptin are reliable markers of nutritional status and play important roles in mediating the metabolic control of gonadotropin secretion [125]. Leptin, a protein hormone synthesized and secreted primarily by adipocytes, has been investigated extensively as a metabolic signal for the control of reproductive neuroendocrine function in various animal species, including in cattle [126]. Circulating concentrations of leptin correlate positively with adiposity [127] and increase with elevations in body weight [128]. In young prepubertal heifers, there is a linear increase in plasma concentrations of leptin as puberty approaches, with increasing body weight and adiposity (body condition score). However, marked changes in leptin synthesis and secretion in blood in cattle are related most strongly to type of diet fed and directional changes in energy balance. High concentrate diets (e.g. high in soluble carbohydrate or fat), which are ruminally propionergic and gluconeogenic in ruminants, create rapid and sustained increases in circulating concentrations of leptin in heifers and mature cows relative to isocaloric high forage diets [129]. Cessation of the high concentrate diet results in an abrupt decline in circulating leptin to levels similar to those observed in cattle on a high forage diet. Moreover, fasting causes an acute decline in circulating leptin [107–109]. However, chronic undernutrition in ruminants results in adaptation and plasma leptin tends to return to values similar to those of full-fed, non-obese cattle on maintenance diets [130]. Thus, the direction of body weight change has a greater influence than body weight or adiposity per se. When full-fed cattle are treated with exogenous leptin, no changes in gonadotropin secretion or metabolic hormones are observed [107]. However, if cattle are fasted for at least 48 hours, they become hypersensitive to leptin. Treatment with recombinant leptin prevents the fasting-induced decrease in LH pulsatility in peripubertal heifers [109], and enhances secretion of LH in mature cows [107] by acting at both the hypothalamus to increase the amplitude of GnRH pulses [131] and the adenohypophysis to increase sensitivity to GnRH [132].

Mediators of leptin actions include neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons in the arcuate nucleus. These neurons contain leptin receptors [133]. NPY is a potent stimulator of feed intake and feed restriction increases expression of the *NPY* gene [134]. The anorexigenic actions of leptin are likely associated with the ability of leptin to decrease *NPY* expression [135]. NPY inhibits GnRH release in cows [5] (Figure 24.6). In contrast to its inhibitory effects on *NPY* gene expression, leptin enhances *POMC* expression [136]. One of the peptide products of the *POMC* gene, α-melanocyte-stimulating hormone (α-MSH), has been shown to also exert direct stimulatory effects on

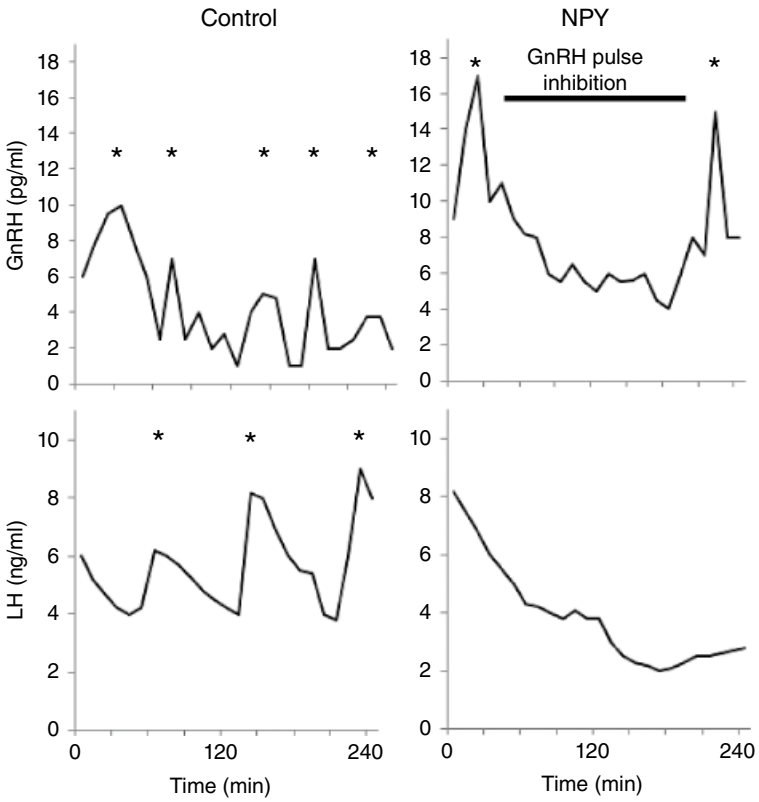


Figure 24.6 Neuropeptide Y (NPY) inhibition of release of GnRH in cows. The pulsatile release of GnRH (top panel) and LH (lower panel) is inhibited following intracerebroventricular administration of NPY (panels on right). Asterisk denotes detected pulses of GnRH and LH. *Source:* From [5], © 1998, Oxford University Press.

GnRH neurons [137]. Therefore the actions of leptin on stimulating GnRH secretion appear to involve suppression of the inhibitory actions of NPY and stimulation of excitatory effects of α-MSH (Figure 24.6, right panel).

Pubertal Development

Puberty is the process through which individuals acquire reproductive maturity. It involves physiological and behavioral changes that are largely driven by activation of gonadal function. In mammalian females, activation of the hypothalamic–adenohypophyseal axis, and the downstream effects of gonadotropins on gonadal function, support enhanced folliculogenesis and steroidogenesis (primarily estradiol synthesis) and lead to first ovulation [138]. The onset of puberty is characterized by an increase in the episodic release of LH, resulting from a heightened frequency of GnRH release and stimulation of gonadotropes [139]. Interestingly, exogenous estradiol induces a surge-like release of LH in prepubertal females [140, 141], indicating that estradiol positive feedback is functional before reproductive maturation is complete. Therefore a major limiting factor for the onset of puberty is the lack of high-frequency episodic release of LH necessary for enhanced estradiol synthesis that will lead to the preovulatory surge of GnRH and LH release.

During the prepubertal period, estradiol negative feedback inhibition of episodic release of LH is enhanced. Ovariectomy in prepubertal heifers, and consequent removal of the major source of estradiol, is associated with a rapid increase in circulating concentrations of LH [45]. Replacement with physiological levels of estradiol in ovariectomized heifers prevents this increase and maintains the pulsatile release of LH at a low frequency. Around the expected time of pubertal transition, the frequency of secretory LH episodes increases to that similar to an ovariectomized heifer receiving no estradiol replacement. Thus, it is hypothesized that decreased sensitivity to estradiol negative feedback during pubertal transition leads to increased episodic release of LH characteristic of the mature state. Although the mechanisms involved in diminished sensitivity to estradiol negative feedback remain unclear, they have been postulated to include changes in estradiol signaling in the hypothalamus [142]. However, a more recent investigation in the ewe lamb using more sophisticated techniques indicated that changes in the

expression of ESR1, particularly in kisspeptin neurons in the arcuate nucleus, failed to explain the pubertal escape from estradiol negative feedback [48]. Also, a population of neurons that synthesize the neuropeptide kisspeptin has been implicated in mediating estradiol's effect on GnRH secretion [143–145]. Kisspeptin is a potent GnRH/LH secretagogue [146, 147]. In addition, estradiol regulates the expression of the gene encoding kisspeptin, likely through direct actions on kisspeptin neurons [143].

Puberty in heifers occurs normally at approximately 10–15 months of age in *Bos taurus* breeds. In *Bos indicus* and *B. indicus*-influenced breeds, puberty usually occurs later and over a wider time range (15–27 months of age) [148, 149]. However, age at puberty is influenced to a considerable degree by nutrition [150]. Undernutrition delays puberty in heifers [104]. In contrast, elevated body weight gain during the prepubertal period advances the onset of puberty [151, 152]. The acceleration of puberty in response to increased weight gain in heifers has been associated with an earlier reduction in the sensitivity to estradiol negative feedback and hastening of the peripubertal activation of the reproductive neuroendocrine axis [153, 154]. Nutritional signals that mediate the nutritional control of pubertal development include nutrients and metabolic hormones. Leptin appears to be a major permissive signal for pubertal progression. In heifers, expression of the leptin gene in the adipose tissue and circulating concentrations of leptin increase as puberty approaches [128]. Among leptin's various functions are its ability to modulate axonal growth, neurogenesis, and synaptogenesis. Changes in leptin signaling during early life initiate changes in the morphology and function of adult hypothalamic circuitries involving nutrition and metabolism [155–157]. The effects of leptin must be placed in context. While fasting-induced suppression of LH pulse frequency in heifers is prevented by treatment with leptin [109], its chronic administration in prepubertal heifers fed a normal diet does not advance puberty [158], supporting observations made in rodents [159] that leptin is necessary but not sufficient to induce puberty in heifers.

Other metabolic hormones, such as insulin and IGF1, can influence neuronal remodeling, including the morphology of GnRH neurons. Therefore nutrition can influence reproductive function by promoting morphological alterations that result in reorganization of neurocircuitries capable of balancing inhibitory and stimulatory inputs to GnRH neurons. These have been shown to include enhanced or decreased numbers of NPY and POMC neurons projecting to GnRH neurons in response to heightened or restricted dietary energy, respectively [156, 157]. Such modifications appear to be long-lasting and may impact lifetime functional phenotype.

Neuroendocrinology of the Postpartum Period

The interval from parturition to resumption of ovarian cycles in the bovine female represents a physiologically distinct and economically critical period of the bovine reproductive lifecycle. Therefore it is not surprising that research to understand the biological basis of postpartum anovulation and to develop strategies for its managerial control has been the subject of intense investigation since the early 1970s [160–165].

In the cow, three main factors contribute to the evolution and maintenance of a state of ovarian quiescence after parturition: (i) a marked depletion of anterior pituitary stores of LH during late gestation, (ii) peripartum nutritional status, and (iii) suppression of high-frequency pulses of GnRH by suckling in the presence of a maternal–offspring bond. Figure 24.7 summarizes the influence of various factors that contribute to the maintenance of anovulation in the postpartum cow and the physiological events during postpartum anovulation.

Depletion of releasable pools of LH within the adenohypophysis occurs in both ewes and cows during the last third of pregnancy and is quite evident at the time of parturition [114–116]. Its basis is the ability of chronically elevated, placental-derived estradiol to suppress anterior pituitary gonadotrope synthesis of the α- and β-subunits of LH and, to a certain extent, FSH [71, 166, 167]. However, this does not appear to be mediated through changes in mRNA expression for the gonadotropin subunits. In experiments with ovariectomized ewes, in which the estrogenic environment of late pregnancy was simulated, pituitary content of both LH and FSH was markedly reduced but abundance of mRNA for the α-subunit or β-subunit of LH and FSH was unchanged or increased. Moreover, treatment with exogenous GnRH in these ewes did not restore pituitary synthesis of gonadotropins. Therefore it was concluded that the negative effects of chronically elevated estradiol are downstream of mRNA expression and represent post-translational regulation of gonadotropin synthesis [71, 166]. In considering this model, it should be noted that in cows neither adenohypophyseal content [167] nor releasable pools [168] of FSH change appreciably during the postpartum period. This may be the result of a more rapid restoration of the β-subunit for FSH by gonadotropes after parturition [169]. Thus, the negative impact of high circulating concentrations of estradiol during gestation in the cow, and its carry-over into the postpartum period, is primarily mediated through effects on LH.

Although progesterone is also elevated during pregnancy, it does not have major direct effects on the pituitary [165]. The main effects of high concentrations of progesterone are

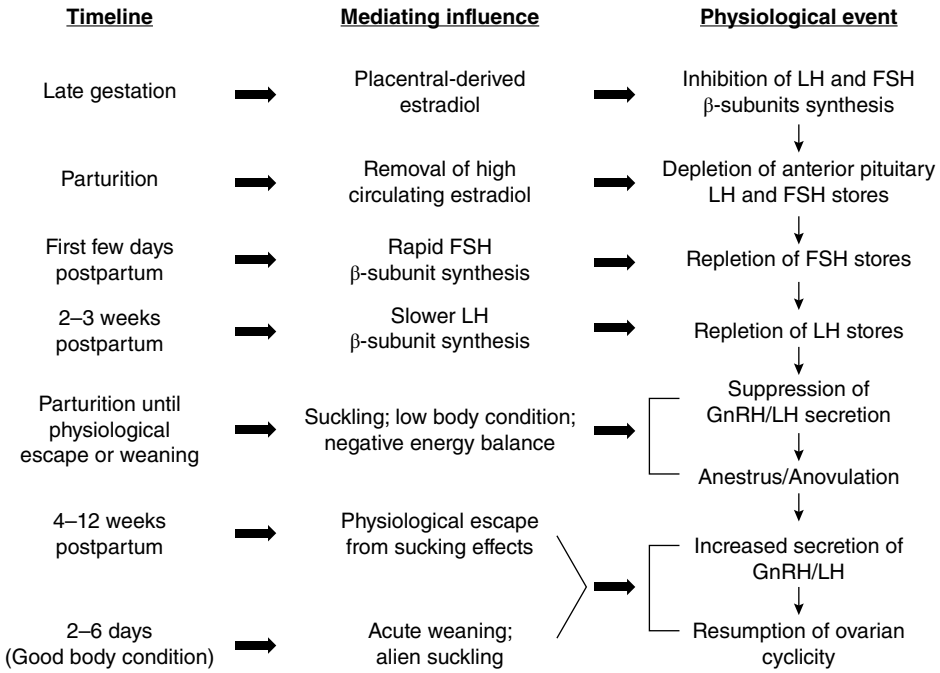


Figure 24.7 Timeline and mediating influences underlying the postpartum anovulatory period of the cow, with emphasis on the suckled beef cow. In the high-producing dairy cow, negative energy balance, as opposed to suckling, is the predominant influence inhibiting reinitiation of estrous cyclicity after calving.

on hypothalamic secretion of GnRH and this inhibition is lifted at parturition. Moreover, hypothalamic content of GnRH does not change from days 1 to 35 postpartum in cows or ewes [165, 170]. Perhaps more important to postpartum recovery is the stimulatory low-level increases in progesterone observed during the resumption of ovarian cycles [171, 172]. Short-lived luteal phases created by premature regression of corpora lutea during the first 30 days postpartum precede the establishment of normal length cycles [173]. In this role, progesterone serves as a positive modulator of postpartum reproduction by priming the entire hypothalamic–pituitary–ovarian–uterine axis for normal gonadotropin secretion [82], normal timing of uterine prostaglandin release [174], and normal luteal function [175–177].

Within two to three days following parturition, the high gestational levels of placental steroids are cleared from the circulation in both cows and ewes [172, 178]. This is accompanied by large increases in the concentration of mRNA for the α- and β-subunits of LH [165]. Within two to three weeks postpartum, total anterior pituitary content and releasable pools of LH are replete in both dairy and beef cows [167, 168, 179]. This process proceeds in the presence of suckling, although the amplitude and/or frequency of GnRH pulses remains low [5]. Thus, in the absence of elevated circulating concentrations of estradiol, the ruminant gonadotrope appears to require very little GnRH stimulus to synthesize and store LH [165]. By 30 days postpartum, release of LH in response to pharmacological challenge with GnRH is notably greater in suckled than in weaned cows [168]. This occurs because the pulsatile release of LH from the pituitary increases dramatically in weaned cows within the first two weeks after calving due to the absence of suckling [163, 180, 181]. Correspondingly, suckled cows accumulate more GnRH within the median eminence [182] and more LH within the anterior pituitary [168] during this period than non-suckled cows. Thus, more LH is available for release in response to GnRH challenge in suckled compared with weaned cows during the first few weeks post calving. Since the repletion of adenohypophyseal LH is essentially complete within three weeks after calving, it would seem reasonable to assume that a normal pattern of LH secretion and onset of ovarian cycles would also occur within this period. However, while first ovulation often does occur within two to three weeks postpartum in milked dairy cows and weaned beef cows, it is delayed for extended and variable periods in the presence of suckling [162, 163]. Suckling frequency must be reduced to no more than once daily in order for duration of postpartum anovulation to be decreased [183, 184]. Moreover, up to six days are required in order for the effects of complete weaning on secretion of LH to result in resumption of ovulatory cycles in a majority of cows [185, 186]. Time of suckling during the solar day had no effect on length of the postpartum anovulatory

interval [187]. However, low body condition at calving and postpartum undernutrition interacts with suckling to exacerbate its negative effects on postpartum reproduction. In some cases, the period of suckling-induced anovulation can extend for 100 days or more due to this interaction, although the average is usually 60–70 days in mature cows calving in good body condition [183, 188]. Of additional interest has been the biostimulatory effect of the bull on resumption of postpartum ovarian function [189, 190]. Data indicate that a pheromonal component of bull urine can hasten the recovery of a pulsatile pattern of gonadotropin secretion in postpartum cows and thus first estrus, although the neuroendocrine mechanism has not been elucidated.

How does the suckling calf influence the neuroendocrine regulation of GnRH and LH secretion? In the 1980s and 1990s, several laboratories around the world focused on this question. Based on historical perspectives in the literature, it was assumed that the suckling calf inhibited the secretion of GnRH and/or the gonadotropins through neural signals mediated by tactile stimulation of the teat [191, 192]. Early endocrine-related work focused on the potential roles of neurohumoral mediators in this process, including prolactin [172, 193, 194] and cortisol [194, 195]. It is now clear that neither of these hormones accounts for the suppressive effects of suckling on the hypothalamic–pituitary axis of cows during the postpartum period. Additional studies addressed the role of ovarian estradiol. Results indicated that enhanced sensitivity to estradiol negative feedback plays a role in mediating the suckling effect [196, 197]. However, estradiol negative feedback is not required for the inhibitory effects of suckling to be manifested [198]. Concomitant with this effect is an apparent increase in endogenous opioid tone that decreases as the postpartum interval increases and in response to weaning [199, 200].

In order to define more clearly whether physical manipulation of the teat alone could account for the inhibitory effects of suckling on gonadotropin secretion, a series of experiments were conducted to examine the effects of various exteroceptive stimuli. These included chronic milking by hand as well as mechanical, electrical, and thermal stimulation of the teats of ovariectomized cows. None of these stimuli was able to recreate or simulate the suckling effect [194, 201–203]. Contrasting studies attempted to simulate the "weaning effect" through mechanical masking of the udder during suckling [203] or by using either complete mastectomy or mammary denervation [204, 205]. Neither approach altered gonadotropin secretion in cows suckling their own calves. Mammary denervation did impair oxytocin secretion and milk let-down following suckling, but this physiological deficit was only temporary. However, denervation had no effect on secretion of prolactin or milk production in beef cows. Collectively, the foregoing studies resulted in the conclusion that chronic tactile stimulation of mammary somatosensory pathways is not required in order for the suckling effect to be manifested [205], and spawned a new avenue of research to assess the role of maternal behavior as a regulator of postpartum anovulation.

Initial experiments involving the role of maternal behavior demonstrated unequivocally that if a cow is forced to suckle an "alien" calf under controlled conditions, the neuroendocrine system responds as if the cow has been weaned: pulsatile secretion of LH increases and first postpartum ovulation occurs within two to six days [186]. This led to experimental observations that cows must be able to identify the calf as "own," using either vision or olfaction, in order for suckling to inhibit the GnRH pulse generator and to sustain the anestrous state [206]. Elimination of both senses prevented calf identification and the negative effects of suckling by the cow's own calf on the reproductive neuroendocrine system. At the neuroendocrine level, suckling appears to suppress secretion of LH by dampening the amplitude of individual GnRH pulses (based on measurements of GnRH secretion in third ventricle cerebrospinal fluid), rather than reducing the number of detectable pulses. This has been interpreted to mean that the number of biologically effective GnRH pulses reaching the adenohypophyseal portal vessels is reduced, thus lessening the frequency of LH pulses secreted by the anterior pituitary [5].

In addition to fundamental studies, a plethora of applied studies have been reported using temporary weaning, alien suckling, and limited suckling to hasten the timing of first postpartum estrus and ovulation, and to enhance synchronization of estrus and ovulation. A detailed consideration of this topic is beyond the scope of this chapter. The reader is referred to published reviews [162–164, 207] and other reports that consider the topic in greater detail.

Novel Neuroendocrine Regulators of GnRH Neuronal Function

An increasing number of neuropeptides have been investigated for their potential roles in controlling reproductive function. In addition to those discussed earlier (in sections "Hierarchical Control of Gonadotropin Secretion: The GnRH Pulse Generator" and "Differential Control of Gonadotropins: FSH"), arginine-phenylalanine-amide-related peptide 3 (RFRP-3), now referred to as gonadotropin-inhibitory hormone, has received great attention [208, 209]. This peptide is synthesized in the hypothalamus and projects to GnRH neuronal cell bodies and/or terminals in the median eminence,

where it has been shown to exert direct control on GnRH neuronal activity and GnRH secretion in rodents, cattle, and sheep. However, reports demonstrating regulatory effects of RFRP on GnRH/LH secretion have been countered by a number of others indicating failure of its ability to exert such effects in the ewe and the mare [210–212].

Although studies investigating the physiological actions of RFRP in cattle are limited, evidence from studies in cattle [213] and other species [214–217] indicate that RFRP-3 acts as an inhibitor of LH release by potential actions in the hypothalamus and at the level of the pituitary. The avian homolog of the mammalian RFRP-3 was the first peptide of the family to be isolated in vertebrates [214]. Because the avian peptide inhibited LH synthesis and secretion [214–216], it was named GnIH. In mammals, the gene that encodes RFRP-3 can produce more than one bioactive peptide of the RFRP family [217]. Expression of the RFRP gene has been localized in the paraventricular and dorsomedial nucleus of the hypothalamus of ewes [50]. Neuronal fibers containing RFRP immunoreactivity are observed to extend to the median eminence, where they are in close proximity to GnRH-containing fibers [215, 216]. Neuronal projections containing RFRP are also observed in close proximity to GnRH cell bodies and proximal dendrites [50]. Thus, RFRP-3 may control gonadotropin release by acting on GnRH neurons and/or at the level of the gonadotrope following its release into the hypothalamic–hypophyseal vasculature. The presence of GPR147, the receptor for RFRP-3, in the hypothalamus and adenohypophysis supports RFRP-3 actions at both levels. Studies in sheep [218] and pigs [219] indicate that RFRP-3 inhibits the release of LH *in vivo;*. However, controversy on the effects of RFRP-3 exists because studies in sheep [210], pigs [220], rats [221], and horses [182] failed to support a clear effect of RFRP. Nevertheless, the observation that RF9, a putative RFRP antagonist, increased gonadotropin release in laboratory rodents [183, 184], sheep [179], and mares [212] suggested that RFRP-3 might function within a complex biological system to effect regulation of gonadotropin release. However, more recent reports have refuted this experimental approach because RF9, the putative ligand for the RFRP receptor (NPFFR1), has been shown to function primarily as a KISS1R agonist, the receptor for kisspeptin on GnRH neurons [222].

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25

Ovarian Follicular and Luteal Dynamics in Cattle

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Introduction

The intent of this chapter is to provide an overview of our present understanding of ovarian function in cattle, with emphasis on the dynamics and control of follicular and luteal gland development. The following sections are arranged, more-or-less, in a chronological order, beginning with fetal ovarian development and follicle assembly, early folliculogenesis and oogenesis, antral follicle dynamics during the estrous cycle, ovulation, and development of the corpus luteum (CL). A following section includes a description of ovarian dynamics during different reproductive states (i.e. pre- and peripubertal periods, during pregnancy and the postpartum period, and during old age). Lastly, reference is made to implications of our new-found knowledge of ovarian function on clinical and breeding management practices in cattle, particularly on the design of modern protocols for ovarian synchronization and superstimulation.

Ovarian Development and Follicle Assembly

The gross, ultrasonographic, and histologic morphology of the mature bovine ovary is illustrated in Figures 25.1 and 25.2. Similar to most mammalian species (except the horse), the bovine ovary is arranged with an outer cortex containing the transient CL and thousands of follicles at differing stages of development, and an inner medulla composed primarily of connective tissue, vessels, nerves, and the rete ovarii.

The ovarian primordium develops from an elongated gonadal ridge on the ventral aspect of the embryonic kidney (mesonephros). Recent immunohistochemical studies of bovine ovaries suggest that the ovarian primordium does not initially have a distinct surface epithelium with a basement membrane separating it from the underlying stroma, but instead is composed of a cluster of "gonadal ridge epithelial-like" cells (GREL) (Figure 25.3) [1]. The GREL cells likely develop from the surface epithelium of the mesonephros and are precursors of ovigerous cords, granulosa cells of future follicles and the surface epithelium of the future ovary [2]. Primordial germ cells (PGC) migrate from the yolk sac/developing hind-gut to the dorsal celomic wall of the embryo via the dorsal mesentery to colonize the gonadal ridge. The PGC undergo massive mitotic proliferation and intermingle with the resident GREL cells. Invading fingers of stroma ("cell streams") from the underlying mesonephros penetrate the gonadal ridge/ovarian primordium and separate the clumps of PGC and associated GREL cells into ovigerous cords that radiate from the surface epithelium toward what will become the ovarian medulla at the base of the ovary [3, 4]. The developing medulla contains stroma, blood vessels, and mesonephric tubules that will persist in the adult ovary as the rete ovarii. The penetrating stroma has a basal lamina at its leading edge, hence the ovigerous cords become surrounded by a basal lamina. As the ovary develops, the ovigerous cords break down into smaller groups of PGC/GREL cells, resulting in the formation of primordial follicles containing an oocyte surrounded by flattened granulosa cells separated from surrounding tissue by a basal lamina. After forming a primordial follicle, mitotic division of the oogonium ceases, it enlarges, and it becomes arrested in the early stages of meiosis I. The first follicles form close to the medulla, while the outer cortex still contains shorter ovigerous cords. The gradual progression of follicle formation extending from the medulla to the surface has been observed in fetal ovaries of human, cattle, and sheep, and in postnatal rat ovaries (reviewed in [1]). At later stages of ovarian development, when preantral and antral follicles appear, the cells on the ovarian surface become single-layered and the stroma

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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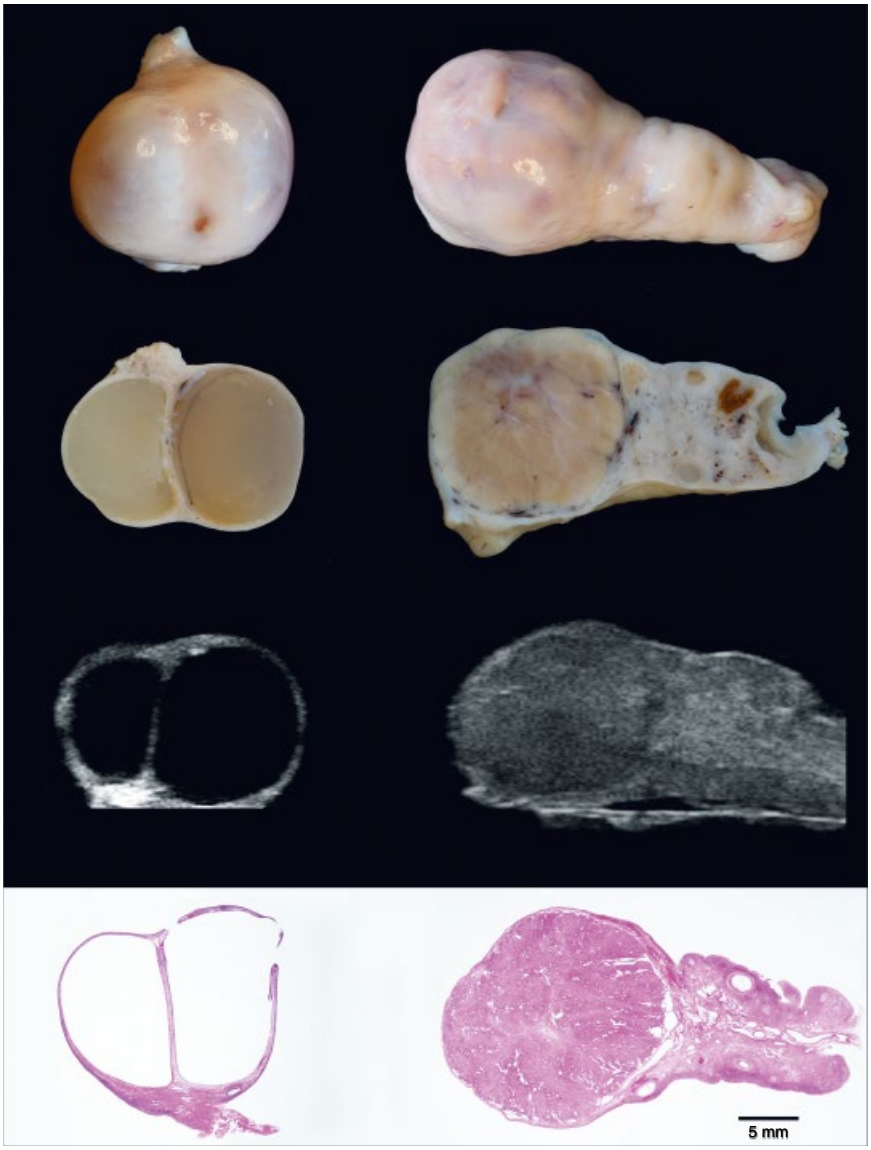


Figure 25.1 Left and right ovaries from a cow in proestrus (top to bottom: surface view, cut surface, ultrasound image, and histological section stained with H&E). In the left ovary, two large antral follicles are apparent side-by-side, a regressing former dominant follicle (on the left) and the preovulatory dominant follicle (on the right). In the right ovary, a regressing CL is the predominant structure. The scale bar is 5 mm for all images.

underlying the surface thickens to become the tunica albuginea. Ovigerous cord formation begins around day 79 of gestation in the fetal bovine ovaries, the cords start to break down by 127 days, follicles are formed by 173 days, while ovarian surface epithelium is detected by 234 days of gestation [5].

Folliculogenesis and Oogenesis

Folliculogenesis is the developmental process in which an activated primordial follicle develops to a preovulatory size through the growth and differentiation of the oocyte and its surrounding granulosa cells [6–8]. Ovarian follicles have been classified in various ways based on morphologic characteristics such as the number of granulosa cell layers surrounding the oocyte, the morphologic features of the granulosa cells, the diameter of the oocyte and the follicle, and the absence or presence of a fluid-filled antrum, but in general may be categorized as primordial, primary, secondary, or tertiary (antral or vesicular; Table 25.1) [9, 10, 12].

Initiation of follicular growth, referred to as activation, begins with the transformation of the flattened pre-granulosa cells of the primordial follicle into a single layer of cuboidal granulosa (follicular) cells, at which point the follicle is referred to as a primary follicle [9, 11, 13]. Factors such as increased mTOR1 in the granulosa cell of activating

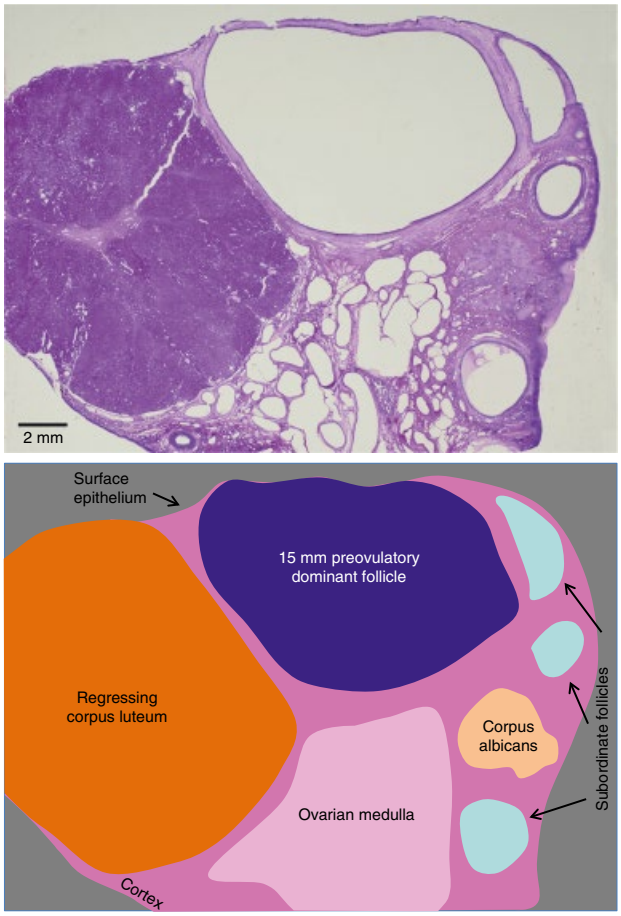


Figure 25.2 Histological section (stained with H&E) and diagram of the ovary of a cow at day 18 of the estrous cycle (day 0 = ovulation) showing structures of the outer cortex (regressing CL, preovulatory and subordinate follicles, corpus albicans) and the inner medulla (rete ovarii, blood vessels, connective tissue).

primordial follicles have been proposed to activate KIT signaling in the oocyte, resulting in activation of the PI3K pathway which is normally kept inhibited by PTEN [14, 15]. It is clear that both granulosa cells and the oocyte participate in the activation process, but the exact mechanism is far from clear at the present time. Proliferation of granulosa cells results in an increase in the number of layers around the oocyte. A follicle with two to six layers of granulosa cells is referred to as a secondary follicle, and a follicle with more than six layers of granulosa cells and a fluid-filled antrum is referred to as a tertiary (or antral) follicle [9, 10]. The diameter of a primordial follicle is about 0.04 mm and the diameter of the smallest antral follicle is 0.25 mm [10]. The largest antral or Graafian follicle becomes the ovulatory follicle following the preovulatory gonadotropin surge [9]. In cattle, primordial, primary, secondary, and tertiary follicles first appear at days 90, 140, 210, and 250 of gestation, respectively [16, 17]. The time required for a follicle to grow from the large preantral stage (secondary follicle) to a mature ovulatory size has been estimated to be about 42 days (Table 25.2; Figure 25.4) [10]. It appears that the growth rate of a follicle changes from slow to rapid after formation of antrum [18].

Antral Follicle Dynamics

While early histologic studies provide insight regarding the morphologic changes that occur during folliculogenesis and an overall impression of the time required, they do not provide an understanding of day-to-day dynamics and control of antral follicle development during the estrous cycle. By inference from Rajakoski's 1960 review [19] of the early literature it appears that two schools of thought prevailed at the time (Figure 25.5): (i) follicular growth and regression is a continuous process and is independent of the phase of the reproductive cycle; or (ii) a reservoir of antral follicles remains in a resting stage, and at a particular time in each species, one or several of these follicles begin to grow and reach maturity during the ensuing estrus. Based on gross and histologic evaluation of ovaries obtained from cows slaughtered on known days of the estrous cycle (n = 1–3 cows/day); however, Rajakoski concluded that for antral follicles (>5 mm) two growth waves occurred during the estrous cycle. The first wave of growth was initiated on the 3rd and 4th days of the estrous cycle (estrus = 1st day) and the second between the 12th and 14th days. His interpretation was that the follicles in each wave that did not ovulate regressed and became atretic.

Contrary to the conclusions of Rajakoski, interpretation of later histological studies supported the notion that follicular growth is continuous and independent of the phases of the cycle [21–23]. Controversy regarding the wave theory of follicular dynamics was evident in later reviews in which the two-wave theory was refuted [24–26]. Evidence was presented to support the concept that follicles are recruited continuously throughout the cycle and the follicle destined to ovulate was a result of coincidence of its stage of maturity (readiness) and the occurrence of the preovulatory gonadotropin surge; i.e. the "propitious moment" theory (Figure 25.5). However, with the introduction of ultrasonography in the late 1980s, the barrier to our understanding of the dynamic process of follicle development was suddenly removed.

The technique of detecting and monitoring ovarian structures by transrectal ultrasonography was described initially in 1984 [27]. By comparing the results of ultrasonography with the results of slicing excised ovaries, the ultrasound technique was validated as a tool for detecting follicles >2 mm in diameter and for monitoring the CL [28, 29]. Subsequent studies using ultrasonic imaging to monitor follicle populations in different size categories [30] or to

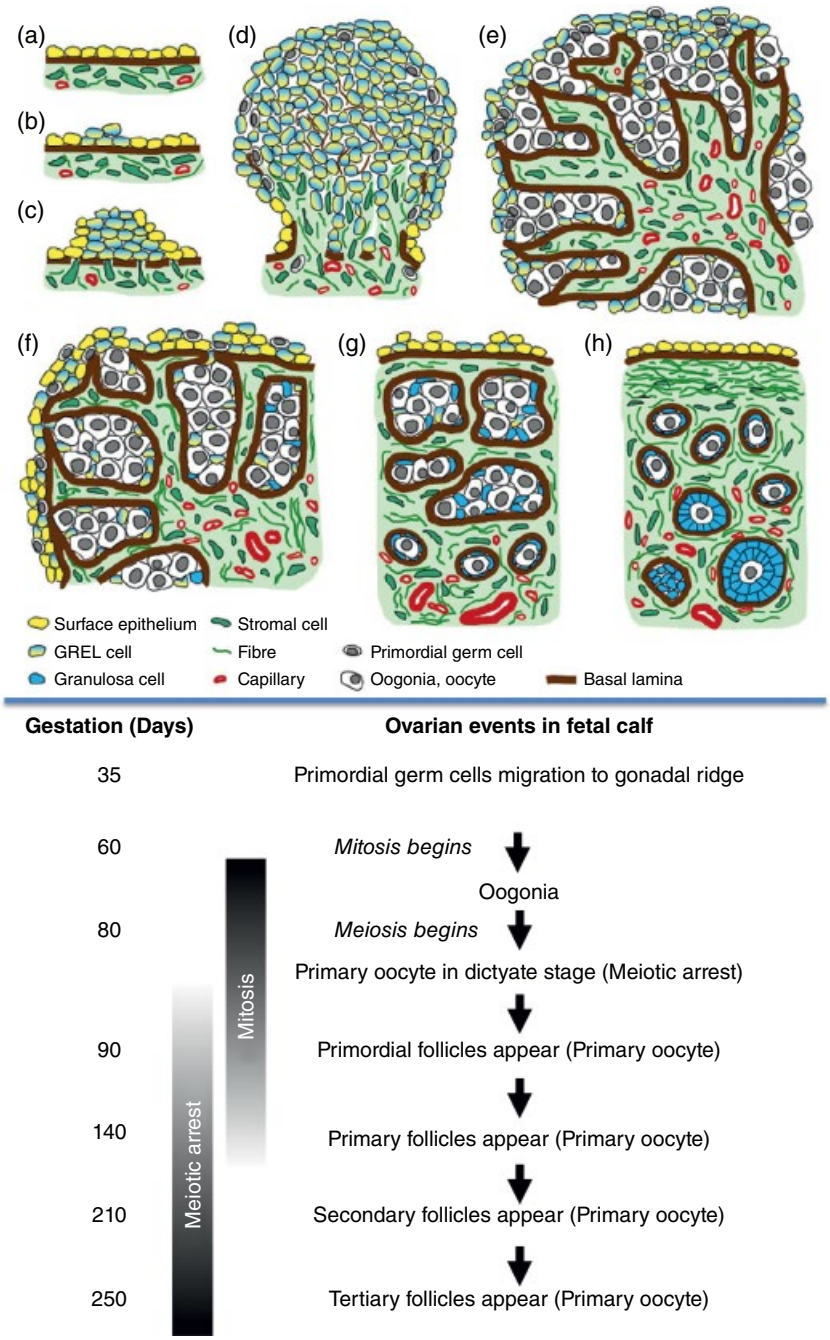


Figure 25.3 (*Above*) Model of bovine fetal ovarian development and follicle assembly and (*below*) chronology of early folliculogenesis and oogenesis. (a) Ovary development commences at the mesonephric surface epithelium (yellow) in the location of the future gonadal ridge. (b) Some surface epithelial cells change phenotype into GREL cells (yellow-blue). (c) GREL cells proliferate and the basal lamina underlying the surface epithelium breaks down, allowing stromal cells (green) to penetrate into the gonadal ridge. (d) GREL cells continue to proliferate and primordial germ cells (gray) migrate into the gonadal ridge between the GREL cells. The mesonephric stroma, including vasculature (red), continues to penetrate and expand in the ovary. (e) Oogonia proliferate and the stroma penetrates further toward the ovarian surface, thus enclosing oogonia and GREL cells in ovigerous cords. The cords are surrounded by a basal lamina at their interface with the stroma, but are open to the ovarian surface. (f) Compartmentalization into cortex and medulla becomes obvious. The cortex is characterized by alternating areas of ovigerous cords and stroma, whereas the medulla is composed of stromal cells, vasculature, and tubules originating from the mesonephros (rete ovarii). As the stroma penetrates toward the periphery, GREL cells at the surface of the gonadal ridge are aligned by a basal lamina at their interface with the stroma and begin to differentiate into typical ovarian surface epithelium (yellow cells). (g) Ovigerous cords are partitioned into smaller cords and eventually into individual primordial follicles as GREL cells mature to granulosa cells (blue cells) and surround an oogonium. (h) Finally, the surface epithelium becomes mostly single-layered and a tunica albuginea, densely packed connective tissue, develops from the stroma below the basal lamina of the surface epithelium. Some primordial follicles become activated and commence development into primary and preantral follicles. *Source:* [1]. Licensed Under CCBY 4.0.

Table 25.1 Characteristics of bovine ovarian follicles during development.

Follicle stage	Follicle type	FSH/LH receptors	Granulosa cell layers[a]	Granulosa cells per section[a]	Follicle diameter (mm)	Oocyte diameter (μm)	Zona pellucida	Theca interna
Primordial	1		1	<10 flattened	<0.04	30	Absent	Absent
Transitory	1a		Entered growing pool and surrounded by a mixture of flattened and cuboidal cells					
Primary	2	FSHr form (granulosa)	1–1.5	10–40[b] cuboidal	0.04–0.08	31	Absent	Absent
Secondary	3 Small preantral		2–3	41–100	0.08–0.13	50	Begins to form	Begins to form
	4 Large preantral	LHr form (theca)	4–6	101–250[c]	0.13–0.25	69	Partially formed	Partially formed
Tertiary (antral)	5 Small antral		>6	>250	0.25–0.5	93	Fully formed	
	Large antral Graafian	LHr in granulosa of dominant follicle		40×10^6	10–15	132		

Source: Adapted from [9–11].
[a] Largest cross-section of the follicle defined by the nucleolus of the oocyte.
[b] Oocyte commenced growth when there were at least 40 granulosa cells in the largest cross-section (fourth generation of follicle cells).
[c] Beginning of antrum formation was observed in follicles with at least 250 granulosa cells in the largest cross-section.

Table 25.2 Developmental rates of bovine ovarian follicles.

		Time in each size class			
Follicle size range (mm)	**Granulosa cell generation**	**Hours**	**Days**	**Percentage atretic**	**Mitotic activity**
0.13–0.28	6	365	15.1	1.6	
0.29–0.67	8	285	11.9	6.6	
0.68–1.52	10	100	4.2	40.5	Maximum
1.53–3.67	11	83	3.5	30	
3.68–8.57	12	186	7.8	67.4	
>8.57	13			60	
Total		1019	42.5		

Source: Adapted from [10, 18].

monitor individually identified follicles [31–34] convincingly documented that follicular growth in cattle occurs in a wave-like fashion and that the majority of estrous cycles in cattle are comprised of two or three such waves. The method by which individual follicles may be monitored throughout one interovulatory interval by ultrasonography is illustrated in Figure 25.6. During daily examinations, a sketch of each ovary is made to record the number, diameter, and relative positions of follicles and the CL. Despite that the ovaries are quite moveable at the end of the mesovarium, their position remains remarkably consistent from one examination to the next, allowing accurate "mapping" of ovarian structures over time. The results of ultrasound studies are consistent with those in which three waves of follicular growth were hypothesized [35] based on the presence of one "estrogen-active" follicle during three different periods of the estrous cycle [36, 37] and increases in estradiol in blood from the utero-ovarian veins during approximately the same time periods [38].

Follicular Waves During the Estrous Cycle

Follicular wave emergence in cattle is characterized by the sudden (within two to three days) growth of 8–41 small follicles that are initially detected by ultrasonography at a diameter of 3–4 mm (Figures 25.6 and 25.7) [30, 33, 34, 39–42]. The growth rate is similar among follicles of the wave for about two days, after which one follicle is then *selected* to continue growth (dominant follicle) while the remainder become atretic and regress (subordinate follicles). In both two- and three-wave estrous cycles, emergence of the first follicular wave occurs consistently on the day of ovulation (day 0). Emergence of the second wave occurs on day 9 or 10 for two-wave cycles, and on day 8 or 9 for three-wave cycles. In three-wave cycles, a third wave emerges on day 15 or 16. Under the influence of progesterone (e.g. diestrus, pregnancy), dominant follicles of successive waves are anovulatory and undergo atresia [43]. The dominant follicle present at the onset of luteolysis becomes the ovulatory follicle, and emergence of the next wave is delayed until the day of the ensuing ovulation. The CL begins to regress earlier in two-wave cycles (day 16) than in three-wave cycles (day 19), resulting in a correspondingly shorter estrous cycle (20 versus 23 days, respectively). Hence, the so-called 21-day estrous cycle of cattle exists only as an average between two- and three-wave cycles.

Two-Wave vs Three-Wave Patterns

The majority of bovine estrous cycles (i.e. >95%) are composed of either two or three follicular waves [44, 45] (Figure 25.7). Some have reported a preponderance (>80%) of either the two-wave or the three-wave pattern during an interovulatory interval, while others have reported a more even distribution. There appears to be no breed- or age-specific predilection for a given wave pattern in *Bos taurus*. An increase in the proportion of three-wave patterns has, however, been associated with a low plane of nutrition [46, 47] and heat stress [48, 49]. In *Bos indicus*, no seasonal effect on wave pattern was detected [50], but the pattern was influenced by parity. The majority of Nelore heifers (65%) exhibited a three-wave pattern, whereas the majority of cows (83%) exhibited a two-wave pattern [51]. Others have reported that up to 27% of estrous cycles in *B. indicus* cows consist of four waves of follicular development, compared to only 7% in *B. indicus* heifers (reviewed in [52]).

Predictive factors associated with a two- versus three-wave pattern may provide insight into mechanisms controlling the pattern, and have important implications for breeding management and the development of effective protocols for ovarian synchronization, superstimulation, and fixed-time artificial insemination. Pregnancy rates in

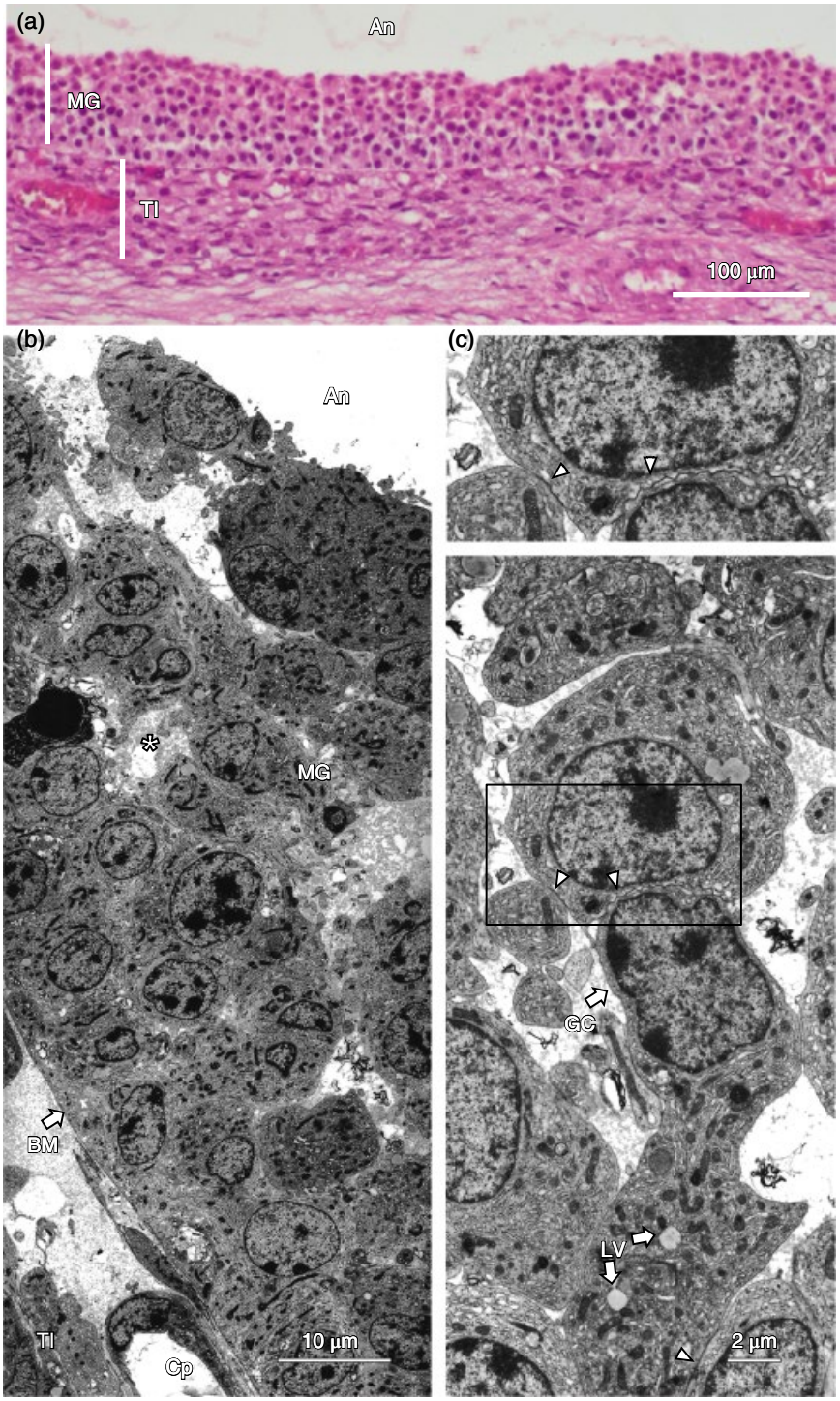


Figure 25.4 Histological section (stained with H&E) and transmission electron micrograph of the wall of the dominant follicle during (a, b) the growing phase (three days after ovulation) and (c) the early static phase (six days after ovulation, right) in cattle. Antrum (An), basement (BM), intercellular spaces (*), membrana granulosa (MG), granulosa cells (GC), theca interna (TI), capillary (Cp). The granulosa cells of the growing dominant follicle have many cytoplasmic processes projecting into intercellular spaces. In the early-static dominant follicle, most of the granulosa cells are polyhedral (others are elongated) and the intercellular spaces are wide. The granulosa cells contain abundant mitochondria, smooth- and rough-endoplasmic reticulum, and occasional lipid vacuoles (LV). The area enclosed in the box is magnified in the upper right corner to show that gap junctions (arrowheads) between the cells are frequent.

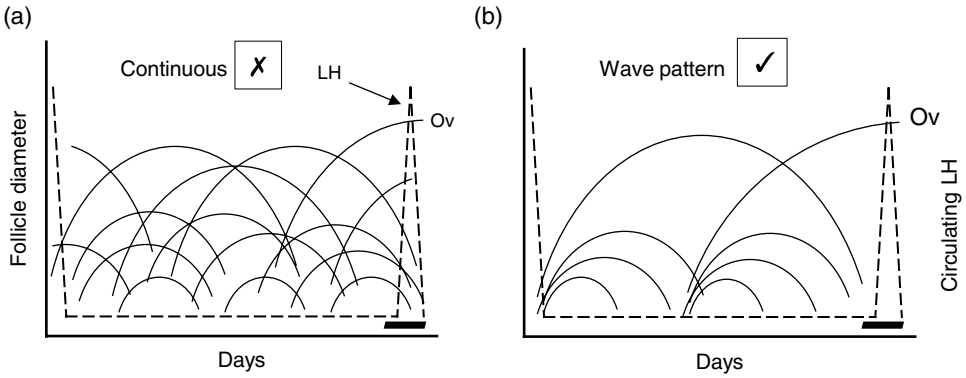


Figure 25.5 Theories of ovarian follicular dynamics during the estrous cycle in cattle, illustrated from one ovulation to the next (i.e. one interovulatory interval). Estrus is indicated by the solid bar along the x-axis and plasma LH concentrations by the dashed line. (a) The theory of continuous recruitment or "propitious moment theory" is that antral follicles grow and regress continuously throughout the interovulatory interval and the ovulatory follicle is the one that by happenstance is at the appropriate stage of development to respond to the preovulatory surge of LH. (b) The wave theory of follicle recruitment is that two or more cohorts (i.e. waves) of antral follicles are recruited (i.e. emerge) during the ovarian cycle, regardless of the presence of a CL (follicular phase or luteal phase). The dominant follicle that develops in the final wave of the interovulatory interval ovulates while preceding waves are anovulatory. *Source:* Modified from [20].

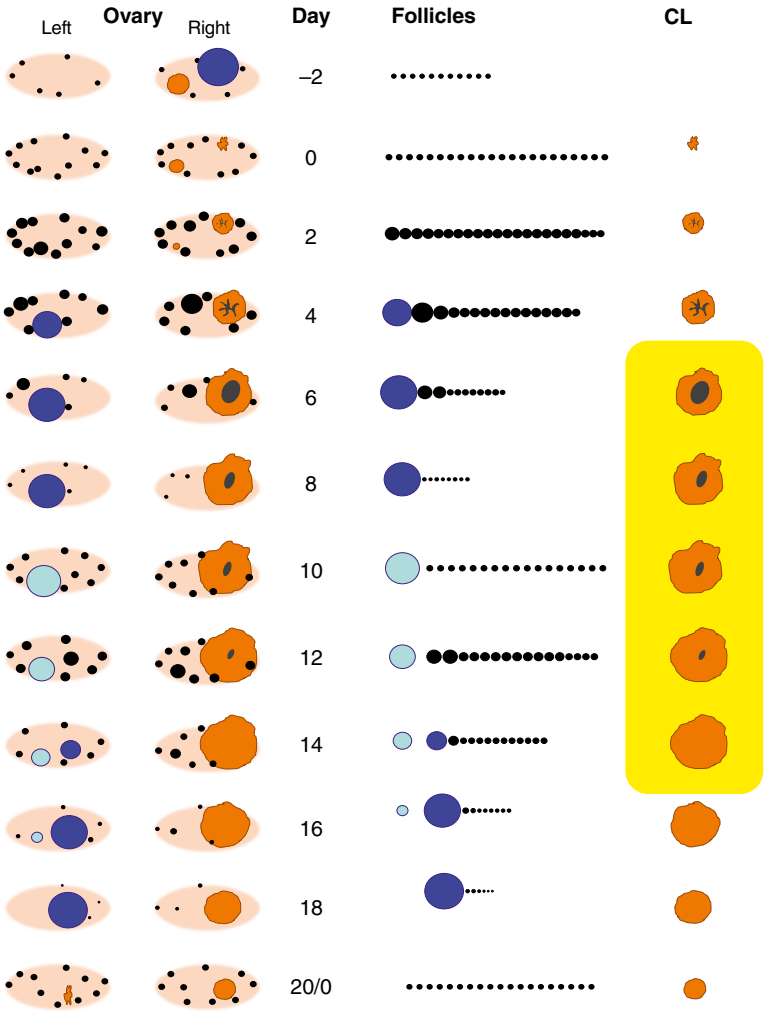


Figure 25.6 Mapping ovarian follicular and luteal dynamics in cattle by serial ultrasonography. During daily examinations (day 0 = ovulation), a sketch of each ovary is made to record the number, diameter, and relative positions of follicles and the CL. The data are used to identify the number of follicular waves during the interovulatory interval (two waves in this example), follicular wave emergence (days 0 and 10), the dominant follicle of each wave (growing: dark blue; regressing: pale blue), the preselection and subordinate follicles of each wave (black), the occurrence of ovulation (days 0 and 20), and the growth and regression of the CL (dark yellow). Note that both ovaries contribute to a given follicular wave and successive dominant follicles may be in either ovary (random). The size and number of ovarian structures in each sketch are tabulated under the respective follicle and CL columns. The high progesterone (luteal) phase is outlined in the yellow box.

cattle with two- versus three-wave patterns were compared [53–55] based on the notion that the preovulatory follicle in the two-wave pattern grows for a longer period [41] and may, therefore, contain a relatively aged oocyte. However, results have been contradictory; pregnancy rates did not differ between two- versus three-wave cycles in some studies [53, 54], whereas a lower pregnancy rate following ovulation at the end of the two-wave pattern was reported in another study [55].

In a more recent study involving ultrasonographic data from 91 interovulatory intervals [44], two- and three-wave patterns of follicular development were compared to determine the repeatability and predictive characteristics of a given wave pattern. Two-wave cycles were three days shorter than three-wave cycles (19.8 ± 0.2 vs 22.5 ± 0.3; $P < 0.01$). The majority of cycles ≤21 days were of the two-wave pattern (88%; $P < 0.05$), while the majority of cycles ≥22 days were of the three-wave pattern (78%; $P < 0.05$). The proportion of successive cycles in which the pattern remained the same (i.e. repeatability) was more than two-fold greater than the proportion of cycles that changed patterns (70% vs 30%; $P < 0.01$). The repeatability of wave pattern and the proportion of two- versus three-wave patterns within the herd were not affected by the season of the year; i.e. they were not affected by pasture-based (spring and summer) versus non-pasture-based (fall and winter) seasons. Interestingly, the strongest correlate to the number of waves in the interovulatory interval was the duration of follicular dominance of wave 1. The duration of dominance of the wave 1 dominant follicle was three days longer and the onset of regression was later in two-wave patterns than in three-wave patterns ($P < 0.01$). Dominance of wave 1 was associated with a subsequent delay in the attainment of maximum diameter by the dominant follicle of wave 2, as well as early onset of luteolysis (Figure 25.7). Results supported the hypothesis that factors that influence the development of the dominant follicle of wave 1 are responsible for regulating the wave pattern.

Two Ovaries – One Wave

The two ovaries act as a single unit; that is, one ovary does not have follicular waves independent of the other. The ovaries respond in unison to factors regulating follicular wave dynamics; hence a follicular wave is the result of multiple follicle growth in both ovaries (Figures 25.6 and 25.7). In a critical study of the intra-ovarian relationships among follicles and the CL [40], the authors concluded that the dominant follicle suppresses subordinates and new wave emergence via systemic rather than local channels. The presence of a dominant follicle in one ovary had no effect that could not be seen equally in the other ovary. Hence, no intraovarian relationships were found in the location of successive dominant follicles during the estrous cycle; i.e. the side of anovulatory and ovulatory dominant follicles was random. These observations are consistent with the findings of early studies on the effects of unilateral ovariectomy. Removal of the ovary with the largest follicle resulted in an increase in follicle development in the remaining ovary, but removal of the ovary without the largest follicle resulted in no such compensatory effect (cited in [40]). While intrafollicular (autocrine and paracrine) factors are important for growth, health, and demise of an individual follicle, there is no convincing *in vivo;* documentation of one follicle affecting the health/regression status of its neighbors directly.

Asymmetry in follicle dynamics in the left and right ovaries has been used to elucidate local versus systemic mechanisms of control of ovarian function, and some have reported greater follicular activity and proportion of ovulations in the right ovary in cattle (c. 60%) [19, 42], whereas others report no such differences [34, 40]. Ovarian follicular asymmetry has also been attributed to the presence of a CL. A positive intra-ovarian effect of the CL on the development of small antral follicles (≤3 mm) has been documented in sheep [56] and cattle [42], but the positive effect did not extend to large dominant follicles [40, 42]. To the contrary, the CL of pregnancy has been associated with a negative intraovarian effect on the dominant follicle [40, 57]. Dominant follicles of successive waves were more frequently (75–80%) found in the ovary contralateral to the CL during pregnancy. The cause of the negative local association between the CL and the follicles is unknown, but it may be directly related to the conceptus rather than to the CL. Support for this notion is found in the observation that there were no ovarian asymmetries during the first two follicular waves in pregnant cattle or in ten successive follicular waves in unmated progesterone-treated heifers [43]. The latter observation, however, is confounded by the fact that exogenous progesterone did not forestall luteolysis in treated heifers; luteal regression occurred at the expected time for a non-pregnant animal regardless of progesterone treatment. However, an experimental design involving hysterectomy during mid-diestrus permitted direct comparison of the effects of a persistent CL due to hysterectomy or due to pregnancy [58]. Results demonstrated that unilateral attenuation of follicle development was due to the conceptus or the ipsilateral gravid uterine horn, and not the CL.

Hormonal Interplay Controlling Follicular Wave Dynamics

Results of early studies of follicle dynamics gave rise to the hypothesis that the dominant follicle suppresses the growth of the subordinates in the existing wave and suppresses the emergence of the next follicular wave [39, 41]. Support for this hypothesis was provided in a series of studies involving

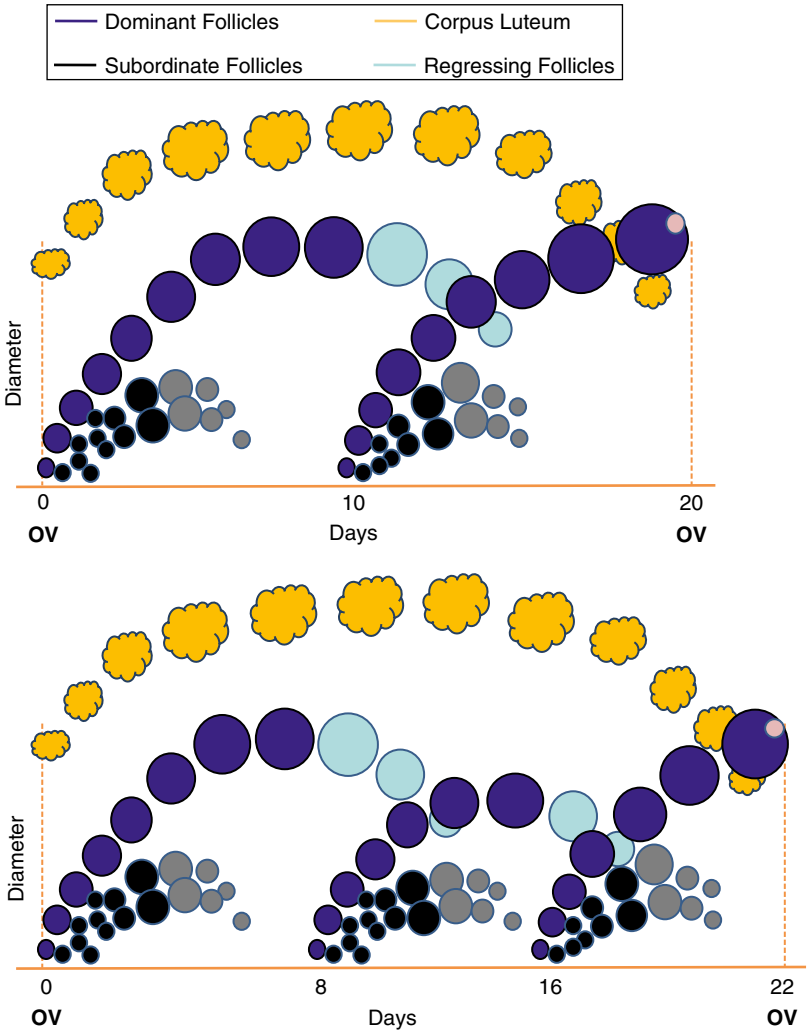


Figure 25.7 Follicular dynamics during two-wave (upper) and three-wave (lower) estrous cycles in cattle. Data from both ovaries are used to identify the dominant follicle of each wave (growing: dark blue; regressing: pale blue), the subordinate follicles of each wave (growing: black; regressing: gray), the occurrence of ovulation, and the growth and regression of the CL (dark yellow). A follicular wave in cattle is characterized by the sudden emergence (within two to three days) of a group of small follicles (n = 8–41) that grow at a similar rate for about two to three days, at which time one follicle is selected to continue growth (dominant follicle) while the rest become atretic and regress (subordinate follicles). The often referred to "21-day" estrous cycle of cattle exists only as an average between two-wave (20 days) and three-wave (22 days) cycles.

systemic treatment with the proteinaceous fraction of follicular fluid and by electrocautery of the dominant follicle [59–62]. The applied implications of these findings were immediate and far-reaching, and marked a new era for ovarian synchronization and superstimulation in cattle (reviewed in [63–65]).

Emergence of a follicular wave and selection of the dominant follicle are temporally and causally associated with a rise and fall in circulating concentrations of FSH [61] (Figure 25.8). Emergence of a follicular wave is preceded by a surge in plasma FSH concentrations in both spontaneous waves and induced waves [61, 67]. Surges in FSH preceding each wave are of similar magnitude; i.e. mid-cycle surges are similar in amplitude and breadth to that of the preovulatory gonadotrophin surge [61, 67]. Follicular products, especially those from the dominant follicle, are responsible for suppressing FSH release and, therefore, the emergence of the next follicular wave [68] (Figure 25.9). A direct relationship between the number of follicles emerging in response to the FSH surge and the subsequent degree of FSH suppression [70–72] documents that all follicles of the new wave contribute to FSH suppression.

Estradiol and inhibin-A and -B are the principal follicular products responsible for suppressing FSH [68, 73–77]. Inhibin-A produced by all the small growing follicles of the wave appears to be the most important suppressor of FSH

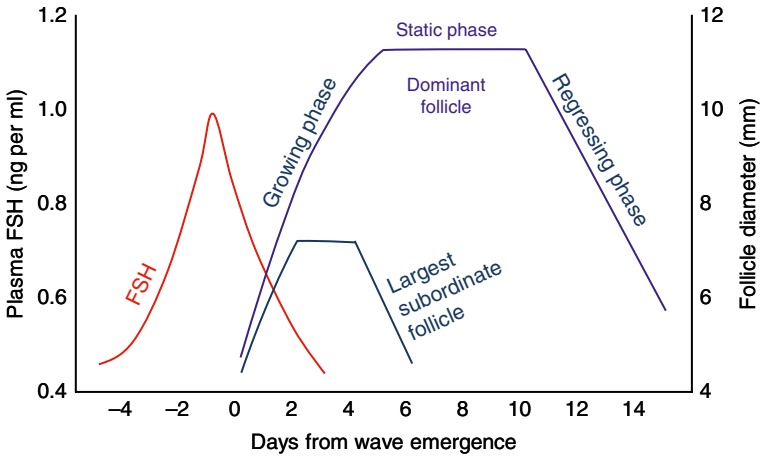


Figure 25.8 Temporal and causal relationship between surges in circulating concentrations of FSH and follicular wave emergence in cattle. Peak concentrations of FSH occur on the day before follicular wave emergence (at 4 mm) and decline to nadir by three days after wave emergence at the time the dominant follicle can be distinguished from the subordinates. Development of a dominant or subordinate follicle may be divided into a growing phase (best characterized by a quadratic equation), a static phase, and a regressing phase (best characterized by simple linear equations). *Source:* Data from [61, 66].

during the first two days of wave emergence, and oestradiol secreted from the dominant follicle is the most important FSH suppressor thereafter [78–81]. The nadir in FSH is reached four days after wave emergence and levels remain low for the next two to three days. At the end of the period of dominance (i.e. at ovulation or the mid-static phase of an anovulatory dominant follicle), circulating concentrations of FSH begin to rise over the next two days and peak about 12–24 hours before emergence of the next follicular wave, when the future dominant follicle is 4–5 mm in diameter. If an existing dominant follicle is removed (i.e. follicular ablation), a surge in FSH begins within the next 12 hours and results in emergence of a new follicular wave within the next 24 hours [59, 82]. Interestingly, FSH rose again only when inhibin-A concentrations reached nadir, despite estradiol declining two days earlier [74].

An inverse relationship between circulating progesterone concentrations and dominant follicle growth was suggested by the observations that (i) in three-wave estrous cycles, the maximum diameter of the dominant anovulatory follicle was greater for wave 1 (under minimal luteal dominance during its growing phase) than for wave 2 (under maximum luteal dominance); (ii) the maximum diameter attained by the dominant anovulatory follicle was greater for wave 1 than for all subsequent waves in pregnant and non-mated progesterone-treated heifers; and (iii) high concentrations of progesterone (endogenous plus exogenous) resulted in a smaller maximum diameter of the dominant follicle of wave 2 compared with subsequent waves (exogenous progesterone only) in non-mated progesterone-treated heifers (reviewed in [83]). A cause-and-effect relationship was established in studies in which progesterone was shown to suppress the dominant follicle during its growing phase in a dose-dependent manner [83] through the negative feedback effect of progesterone on LH pulsatility [84–86]. The suppressive effects on the dominant follicle were not mediated by decreased circulating FSH since progesterone treatment did not suppress FSH [83]. High plasma progesterone during follicular growth resulted in a smaller, shorter-lived dominant follicle and an earlier surge in FSH preceding the next follicular-wave, and hastened emergence of the next follicular wave (Figure 25.10). Conversely, low concentrations of progesterone resulted in an oversized persistent dominant follicle, a delayed surge in FSH, and delayed emergence of the next wave [83]. The oversized dominant follicle in the low-dose group would have been considered a pathological follicular cyst because of its size and persistence for more than 10 days in the absence of a CL [87, 88]. Low-level progesterone exposure (and therefore less inhibition of LH) at the time the dominant follicle normally stops growing appears to be a fundamental component in the etiology of ovarian follicular cysts [83].

Selection of the Dominant Follicle

In monovular species, the term "selection" refers to a process by which a single follicle becomes functionally and morphologically dominant over other follicles of a wave. In cattle, the mechanism of selection of the dominant follicle is based on relative reliance of follicles within a wave on FSH and their relative responsiveness to LH. The transient rise in FSH permits sufficient follicular growth so that some (not all) follicles acquire LH responsiveness. The ability to respond to LH imbues the follicle with the ability to survive without FSH.

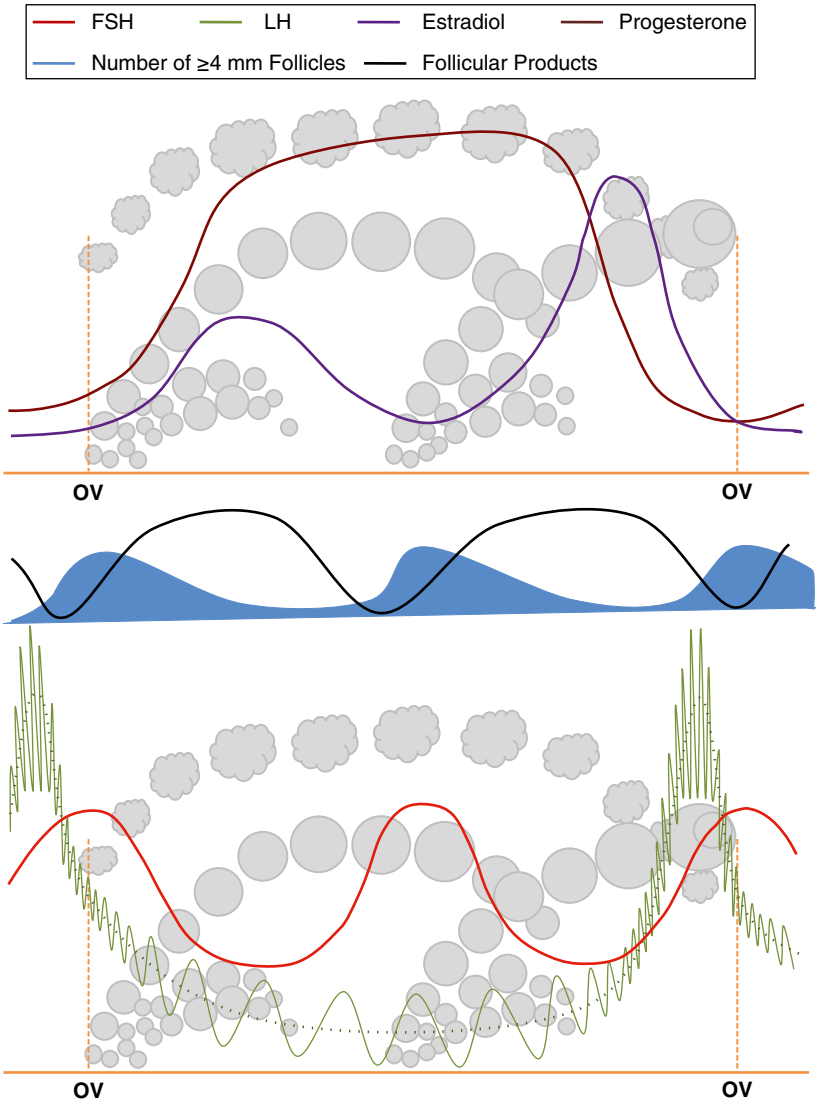


Figure 25.9 Hormonal interplay controlling follicular wave dynamics during a two-wave interovulatory interval in cattle. Dominant and subordinate follicles of each wave are indicated as gray-shaded circles, and the CL is the gray-shaded irregular shape drawn above the follicles. A surge in circulating concentration of FSH precedes emergence of each wave. A surge in circulating concentration of LH precedes ovulation. The LH surge is preceded and succeeded by a period of high LH pulse frequency as a result of low circulating concentrations of progesterone (i.e. period of luteolysis and luteogenesis, respectively). The number of follicles ⩾4 mm fluctuates in a wave-like fashion in response to surges of FSH. Products of the follicles of each wave (primarily inhibin and estradiol) feedback on the hypothalamus and pituitary to suppress FSH. Concentrations of FSH decline at the time that selection of divergence in growth rate of the dominant vs subordinate follicles and follicle numbers decline. The dominant follicle of each wave is the primary source of estradiol and production is twice as high in the ovulatory vs anovulatory dominant follicle. Upon the demise or ovulation of the dominant follicle, the follicular products responsible for suppressing FSH drop and circulating concentrations of FSH are again allowed to surge, resulting in new wave emergence. *Source:* Adapted from [69].

It remains unclear precisely when the process begins, but selection of the dominant follicle is associated with declining levels of FSH in circulation during the first three days of the wave [60, 61]. By using the ablation-replacement approach, a recent study documented that dominant follicle selection in cattle and growth thereafter is due to LH actions [89]. Small follicles of the emerging wave (i.e. <6 mm) are dependent on elevated circulating concentrations of FSH for continued development. In the face of the post-surge decline in FSH, the growth of most of the follicles of a wave stops and they begin to regress within two to five days of emergence. Conversely, the follicle destined to become dominant can maintain cell proliferation and estradiol production despite declining FSH concentrations – an ability that appears to be brought about by maintaining high FSH receptor mRNA expression and

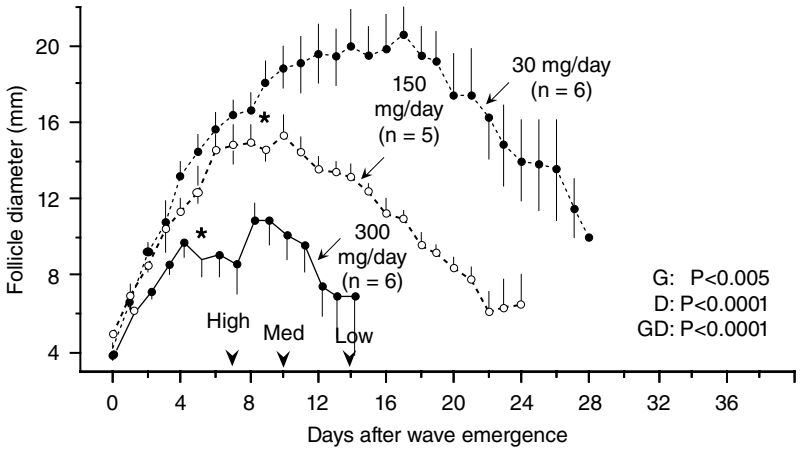


Figure 25.10 Suppressive effect of progesterone on the growth of the dominant follicle in cows. Exogenous progesterone administered in low (30 mg/day), medium (150 mg/day), and high (300 mg) doses for 14 days resulted in progressively greater suppression of the growth of the dominant follicle and progressively shorter interwave intervals (arrowheads on x-axis). Maximum plasma progesterone concentrations were 2, 9, and 17 ng/ml in the low-, medium-, and high-dose groups, respectively. The suppressive effect on the growing phase of the dominant follicle is attributed to progressively more suppression on LH pulse-frequency. P-values from the analysis are include. G, Group effect; D, day effect; GD, group by day interaction. *Source:* Adapted from [83].

FSH-binding affinity [36, 37, 90, 91]. It is becoming clear that the initial exposure to FSH and the ability of the dominant follicle to transition from FSH to LH are critical for the selection of a single dominant follicle in cattle [92].

Divergence in the growth profiles (defined as a significant difference in diameter) of the largest and second largest follicle is a manifestation of the selection process and is apparent by 2½ days after wave emergence (Figures 25.8 and 25.9) [60]. Similarly, follicle "deviation" (defined as a difference in growth rate between the dominant and subordinate follicles) is manifest at 2½ days after wave emergence, when the future dominant follicle reaches a mean diameter of 8.5 mm in Holstein heifers (reviewed in [93]). Continued development of the dominant follicle beyond the 8-mm threshold (point at which divergence in the growth rate of the largest two follicles becomes evident) is associated with a transition from FSH- to LH-dependence [78, 80, 94]. Granulosa cells from the dominant follicle acquire an enhanced ability to bind LH compared to that of the subordinates and have greater LH receptor mRNA expression [37, 90, 91, 95, 96]. Although a slight increase in LH receptor mRNA expression was detected in granulosa cells from the largest follicle a few hours before the onset of deviation [97], it is not yet clear whether the observed increase in LH receptor expression in the dominant follicle is the cause of dominance or a consequence of the selection process. Perhaps the increased LH receptor expression is merely a reflection of a follicle with a developmental advantage before such an advantage becomes ultrasonographically apparent. In this regard, results of a critical study of the development of small follicles support the hypothesis that the follicle destined to become dominant has a size advantage over the others in the wave from its earliest detection at 1 mm (Figure 25.11; see next section on small antral follicles).

Differential production of follicular-fluid factors of the dominant vs subordinate follicles has been associated with the development of greater sensitivity to LH and FSH in the developing dominant follicle, and close temporal coupling between changes in FSH and follicle diameter likely accounts for the increase in growth rate of the dominant follicle before the subordinate follicles can reach a similar stage of development [80]. In this regard, intrafollicular concentrations of IGF-1 remain elevated and estradiol begins to increase in the incipient dominant follicle, while both begin to decrease in the incipient subordinate follicles [98]. Higher concentrations of free IGF-1 in the largest follicle near the beginning of deviation are temporally associated with greater proteolytic degradation of IGF binding protein (IGFBP) -4 and -5 in the largest follicle [99]. Concomitantly, the concentrations of IGFBP-4, -5 [99], and -2 [97] are greater in the second-largest follicle. Not surprisingly, more recent transcriptomic studies have confirmed that major functional pathways in the growth and selection of dominant follicles in cattle involve genes regulating cell differentiation/proliferation and apoptosis/cell death [95, 100–102]. Even when follicle selection is prevented by superstimulatory treatment, multigene bioinformatic analysis confirmed that markers of fertility and follicle maturity are upregulated after longer seven-day FSH treatment than the conventional four-day treatment [103], thereby indicating that duration of follicle differentiation is also a vital factor.

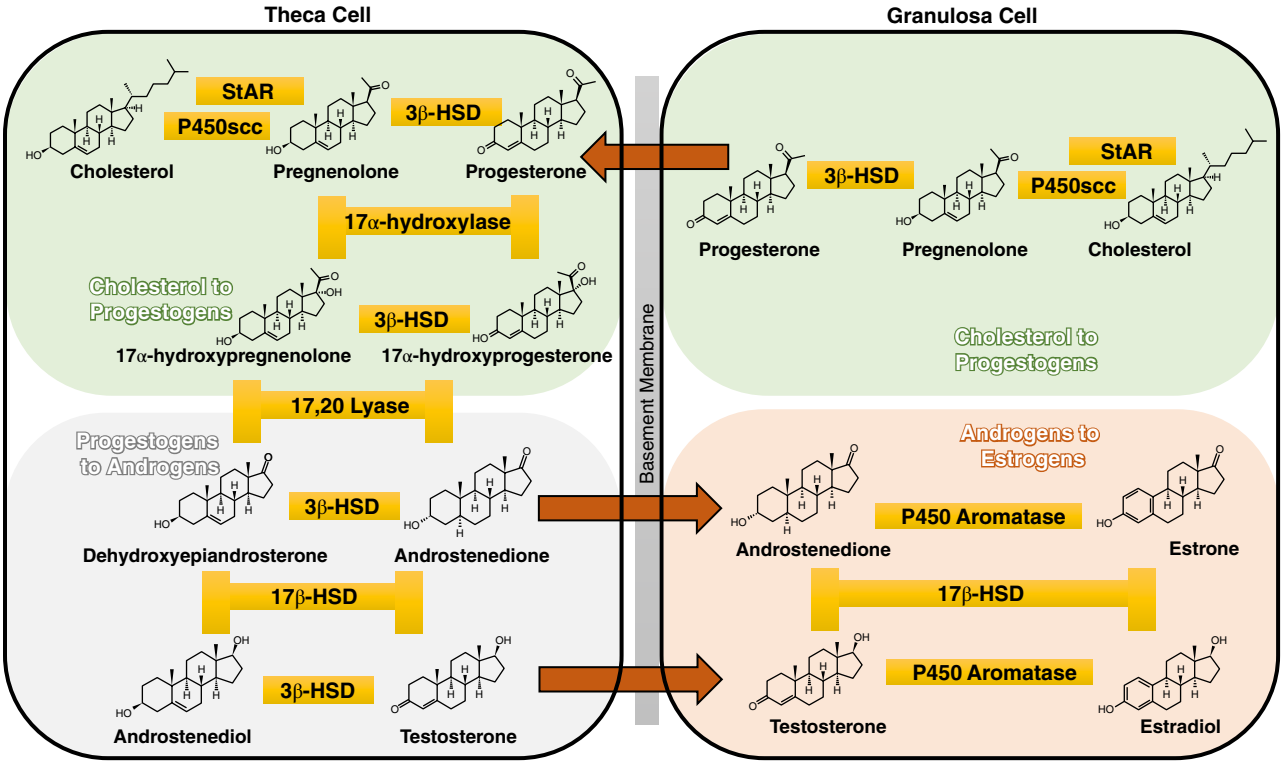


Figure 25.11 Steriodogenesis in ovarian follicles entails the cooperation between granulosa (right) and theca (left) cells and requires P450 side cleavage chain (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase, 17,20-lyase, 17β-hydroxysteroid dehydrogenase (17β-HSD), and P450 aromatase enzymes. Both cell types are capable of producing progestogens from cholesterol. Conversion from progestogens to androgens occurs only in theca cells, while conversion from androgens to estrogens is confined exclusively to granulosa cells.

Co-dominance

Subordinate follicles can achieve dominance if the original dominant follicle is removed [60, 71] or if exogenous FSH is supplied [59]. Similarly, co-dominance (more than one dominant follicle in a wave) is associated with a smaller-diameter profile [59, 104], suggesting competition over limited availability of LH. Double ovulations result from co-dominance during the ovulatory wave. The incidence of co-dominance and double ovulation was low or nil in studies of non-lactating heifers [34, 39, 105–107], but co-dominance ranged from 21 to 36% of follicular waves in lactating dairy cows [107]. A high incidence (10–39%) of multiple ovulations in high-producing dairy cows has been described in several studies [104, 108, 109]. In cows that ovulated multiple follicles, all ovulatory follicles emerged from the same follicular wave [107]. In cows that developed multiple dominant follicles, the process of deviation was associated with lower circulating concentrations of inhibin and progesterone than in cows that developed a single dominant follicle [104]. These effects are consistent with higher circulating concentrations of FSH and LH at the expected time of deviation, continued growth of the second and third largest follicles of the wave, and development of multiple dominant follicles.

Follicular Steroidogenesis – Estradiol Production

Early antral follicles primarily produce proteins and growth factors. Follicular fluid obtained from 1-mm antral follicles contains a very small amount of estrogens, whereas three days later the newly selected dominant follicle (8–9 mm) has >15 times more estradiol than progesterone in its follicular fluid [68]. Steroid production requires a coordinated effort by theca and granulosa cells and is independent of oocyte status. Circulating low-density lipoproteins in blood provide the precursor molecule cholesterol for the biosynthesis of steroids (Figure 25.11). In the granulosa and theca cells, the steroidal acute regulatory protein (StAR) is required to transport cholesterol from the outer into the inner mitochondrial membrane and is one of the key regulatory enzymes in the ovarian steroid pathway. StAR mRNA levels increase with follicular development; levels are highest at the time of maximum size of the dominant follicle and become low or undetectable in atretic follicles [90]. Inside the mitochondria, the conversion of cholesterol to pregnenolone is regulated by the P450 side-chain cleavage enzyme (P450scc; encoded by CYP11A1 gene) in the granulosa and theca cells [110]. Pregnenolone can be converted to either 17α-hydroxypregnenolone by 17α-hydroxylase (encoded by

CYP17A1 gene) or progesterone by the 3-beta-hydroxysteroid dehydrogenase enzyme (3β-HSD). Further, progesterone to 17α-hydroxyprogesterone conversion can be catalyzed by 17α-hydroxylase, while 3β-HSD converts 17α-hydroxypregnenolone to 17α-hydroxypregnenolone. Conversion of progestogens to androgens occurs only in theca cells. Progesterone produced by the granulosa cells must get exported to the theca cells for this conversion to happen. The enzyme 17,20-lyase in the theca cells then coverts 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA) or 17α-hydroxyprogesterone to androstenedione. DHEA can yield either androstenediol by 17β-HSD or androstenedione via 3β-HSD. Both androstenediol and androstenedione can produce testosterone by 3β-HSD and 17β-HSD, respectively. Coversion of androgens to estrogens requires aromatase enzyme ($P450_{arom}$; encoded by CYP19A1 gene) and occurs exclusively in the granulosa cells. $P450_{arom}$ catalyzes conversion of androstenedione and testosterone into estrone and estradiol, respectively. Finally, estrone can be converted to estradiol by 17β-HSD [110, 111]. Successful production of estradiol by a dominant follicle requires the optimal health and balanced effort of both the theca and granulosa layer of the follicle wall.

Small Antral Follicles – When Do Follicles Join a Wave?

Previous ultrasonographic studies have detailed the developmental dynamics of follicles ≥4 mm (described above), but with the availability of newer ultrasound scanners capable of resolving structures as small as 1 mm, the developmental pattern of 1- to 3-mm follicles in cattle has been examined in detail [112]. Results revealed a change in the number of 1- to 3-mm follicles over days, with a maximum one or two days before conventionally defined wave emergence (i.e. when the dominant follicle is first detected at 4 mm), followed three to four days later by a maximum in the number of ≥4 mm follicles. The future dominant follicle was first identified at a diameter of 1 mm and emerged 6–12 hours earlier than the first subordinate follicle ($P < 0.01$; Figure 25.12). After detection of the dominant follicle at 1 mm (0 hours), its diameter was greater than that of the first and second subordinate follicles at 24 and 12 hours, respectively, when the dominant follicle was 2.4 ± 0.17 mm and 1.7 ± 0.14 mm (Figure 25.12). The growth rate of the dominant follicle was greater than that of the first and second subordinate follicles at 120 and 108 hours, respectively, when the dominant follicle was 9.5 ± 0.30 mm and 8.8 ± 0.49 mm. The authors concluded that: (i) 1- to 3-mm follicles develop in a wave-like manner in association with surges in plasma concentrations of FSH; (ii) such follicles are exquisitely responsive to transient elevations in FSH (i.e. within six hours); (iii) selection of the dominant follicle is manifest much earlier than previously documented; (iv) follicular dominance is characterized by a hierarchical progression over a period encompassing the entire FSH surge (five days; Figure 25.12); and (v) divergence in growth rates [93] (referred to as "deviation") of the dominant and largest subordinate follicles is apparent when the largest follicle reaches a diameter of 8–9 mm.

While the developmental dynamics of follicles ≥1 mm have now been well characterized, the dynamics of smaller follicles remain a mystery. An early study demonstrated the binding of FSH to the granulosa of follicles with only a single layer of granulosa cells [113], but it has been argued that they may not be coupled to the adenylate cyclase-second messenger system during early stages of folliculogenesis and may be non-functional [114]. However, the growth-promoting effect of FSH on preantral and small antral follicles in cattle produced *in vitro;* [115] and *in vivo;* [116, 117] suggests a role for FSH in the development of early-stage preantral and antral follicles. These observations challenge the old dogma that early follicle growth is gonadotropin-independent. In this regard, the activation of primordial follicles is thought to be gonadotropin independent.

If small follicles are responsive to FSH, it is logical to postulate that their developmental dynamics follow a wave-like pattern in response to periodic endogenous surges of FSH. Until recently, reference to a follicular wave was limited to follicles ≥4 mm, based simply on the limit of resolution of existing ultrasound equipment. At the microscopic level, there is no morphologic distinction between mid- to late-stage antral follicles <4 mm and those ≥4 mm [9, 10]. At the cellular level, both size categories of follicles not only express FSH receptors but also have a similar level of expression on a per granulosa cell basis (reviewed in [90]). The periodic emergence of waves of follicles ≥4 mm in response to periodic surges in the circulating concentrations of FSH [61] and the consistency in the number of follicles ≥2 mm [118] or ≥3 mm [70] recruited into successive waves indicate that the follicles may become progressively entrained to waves from the earliest stages of development.

Ovulation and Oocyte Maturation

Ovulation involves a series of events that culminate in the evacuation of the antral contents of a follicle, including its mature cumulus-oocyte complex (Figure 25.13), and the initiation of development of a CL. Several events happen in succession and are tightly coordinated at systemic, cellular, and molecular levels. The process begins with endocrine changes triggered by luteolysis. A decrease in circulating concentrations of progesterone at the time of luteolysis

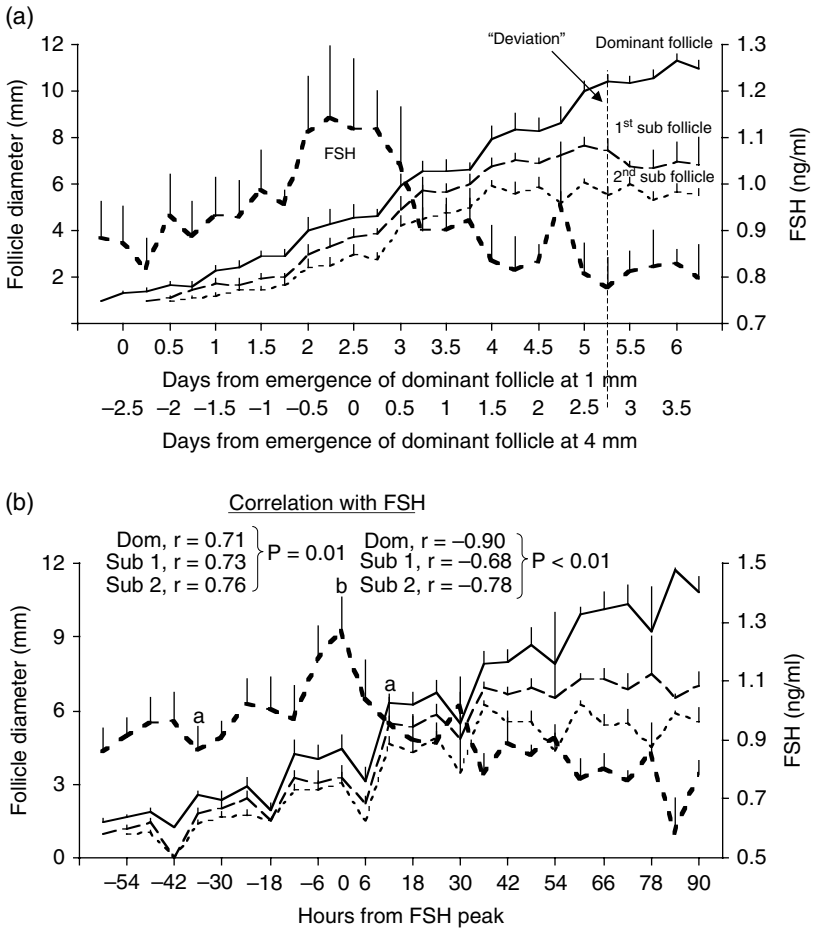


Figure 25.12 Growth (mean ± SEM) of dominant and subordinate follicles in cattle (n = 9) relative to: (a) wave emergence (defined as the day on which the dominant follicle was detected at 1 or 4 mm in diameter; double x-axis), and (b) the peak in plasma FSH concentrations (mean ± SEM). (a) The future dominant follicle had a size advantage over all other follicles of the wave from its earliest detection at 1 mm (much earlier than previously documented). Successive slowing of the growth rate of the third, second, and first largest follicles of a wave during the decline in the FSH surge suggests that the selection mechanism involves sequential suppression of progressively larger follicles over a period of 72 hours (i.e. selection hierarchy). "Deviation" is defined as the first day on which the growth rate of the largest and second largest follicles differed ($P \leq 0.05$). (b) Plasma FSH concentrations were positively correlated ($P = 0.01$) with follicle diameter from the time of follicle detection at 1 mm to the time at which FSH concentrations peaked. Transient elevations in plasma FSH concentration were followed within six hours by an increase in the growth rate of 1- to 3-mm follicles. *Source:* Adapted from [112]. [ab] For FSH, values with no common superscript are different ($P \leq 0.05$).

removes the negative feedback effects on the pituitary and hypothalamus, resulting in an increase in LH pulse frequency and amplitude. Availability of LH results in final maturation of the dominant follicle with an increase in production of estradiol and upregulation of LH receptors on granulosa cells. The preovulatory increase in LH results in extensive ovarian matrix remodeling (vascular changes, disintegration of the basement membrane, and rupture of the follicle wall), granulosa and theca cell responses (cumulus cell expansion, luteinization), and resumption of meiosis by the oocyte. At estrus, the high circulating concentration of estradiol in the absence of progesterone is responsible for a surge release of LH and estrous behavior (increased locomotion, phonation, mounting, and standing to be mounted).

Although LH receptors are present on both granulosa and theca cells [11], receptor distribution within the granulosa cells is confined to the mural granulosa cells. In addition, LH receptors are highly expressed in those layers closer to the basement membrane than those closer to the antrum [119]. Consequently, LH needs second messengers to be able to affect the entire follicle and the oocyte. The effect of second messengers is facilitated by the intimate relationship among granulosa cells and cumulus cells and the oocyte through gap junctions (Figure 25.4) [120]. The two main second messengers for LH are cAMP and intracellular calcium [121]. Binding of LH to the G protein receptors on the granulosa cell surface results in activation of adenyl cyclase, an increase in cyclic AMP, and stimulation

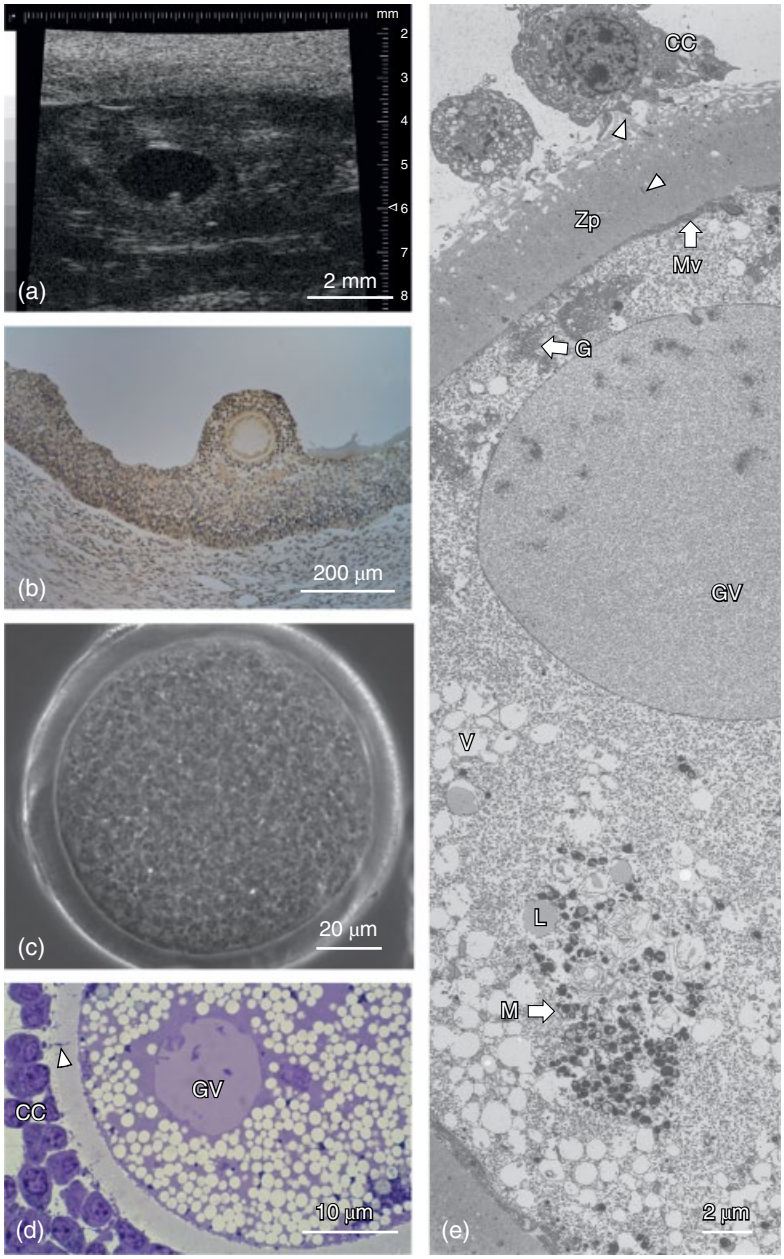


Figure 25.13 Images of the bovine cumulus-oocyte complex (COC). (a) Ultrasound biomicroscopy using a 40-MHz transducer. (b) Histology (5 μm section) stained with Masson's trichrome. (c) Phase contrast microscopy of a denuded oocyte (40×). (d) Semi-thin section (1 μm section) of the COC from a growing phase dominant follicle, stained with toluidine blue. (e) Transmission electron microscopy of (D). Cumulus cells (CC), zona pellucida (ZP), oocyte vesicles (V), lipid droplets (L), hooded and normal mitochondria (M), cumulus cell projections (arrowheads), microvilli on ooplasm surface (Mv), Golgi (G), germinal vesicle (GV).

of the protein kinase A and C pathways. Stimulation of PKA and PKC cause phosphorylation and activation of transcription factors (Egr-1, C/EBPβ, CREB, Fos, Jun, Fra2, JunD) within four hours of the LH surge. Expression of some of these genes is transient, while others are sustained until ovulation (review in [122]). C/EBPβ controls expression of COX-2, which is a key gene in the prostaglandin pathway. Granulosa cell products of epidermal-like growth factor (EGF) expression (epiregulin, amphiregulin, and betarcellulin) are elevated one to three hours after the LH surge and are involved in transmitting the ovulatory signal to cumulus cells [123]. The LH surge also indirectly induces activation of the progesterone and prostaglandin pathways [124] that modify fibroblast function and produce chemokines that recruit inflammatory cells and growth factors that initiate angiogenesis (e.g. VEGF). The LH-induced

extracellular protease ADAMTS1 is synthesized in granulosa cells, but the protein is translocated to the extracellular matrix to induce the production of versican (chondroitin sulfate proteoglycan 2) (reviewed in [125]) and is implicated in the activation of tissue proteases such as the metalloproteinases required for follicle rupture.

The extracellular matrix that surrounds and supports cells is a major component of connective tissue (Figure 25.4). It is composed mainly of structural proteins (elastin and collagens), specialized proteins (fibrillin, fibronectin, laminin), and proteoglycans. It plays a prominent role in ovarian function by participating in processes such as cell migration, proliferation, growth, and demise during follicle development, ovulation, and CL formation. Two families of enzymes govern degradation of the ovarian epithelium during ovulation: metalloproteinases and plasminogen activators. Expression of both of these enzyme families is induced by the LH surge. Changes in gene expression of metalloproteinases and metalloproteinase inhibitors throughout the bovine estrous cycle have not been reported, but one factor that can induce expression is TNFα, which is secreted by preovulatory bovine follicles [126]. Heparanase (encoded by HPSE) cleaves heparan sulfate glycosaminoglycans during matrix remodeling and may also play a major role in follicle rupture during ovulation in cattle [127]. LH-induced ovulation is also associated with the increase in the expression of yet another group of proteases called kallikreins [128]. It appears that many paracrine factors, pro-inflammatory cytokines, and families of protease enzymes act in concert with each other, leading to degradation of extracellular connective tissue and ultimately follicle rupture, on average 30 hours after the LH surge [129].

Meiotic progression of the oocyte is arrested at prophase I (first meiotic arrest) during the fetal development of primordial follicles. The process of resumption of meiosis, release of half the set of chromosomes as a polar body, and progression to metaphase II is referred to as nuclear maturation (Figure 25.14). Shortly after the LH surge, the gap junctions between cumulus cells and the oocyte deteriorate [131, 132]. The loss in communication between cells removes inhibitory signaling and the oocyte can then resume meiosis. However, meiosis is stopped again at metaphase II (second meiosis arrest) until fertilization, when meiosis is finally completed. Mammalian oocytes have the propensity to resume meiosis if they are removed from the antral follicle in which they are housed [133–135], or if they are within preovulatory follicles under the influence of the LH surge [131, 136]. In cattle, a major proportion of oocytes reached MII at 20 hours of *in vitro* maturation [131] or 20 hours after a spontaneous or induced LH surge [136–138]. Cyclic AMP and GMP are considered the primary inhibitors of meiotic resumption in the oocyte [132]. Cyclic GMP produced by granulosa cells is thought to enter the oocyte via gap junctions between the oocyte and cumulus cells to maintain low levels of cAMP phosphodiesterase 3A (PDE3A) in the oocyte [139]. Low PDE3A is thought to maintain high levels of cAMP produced within the oocyte [140] and thus maintain meiotic arrest. Exposure to LH decreases cGMP production by granulosa cells through as yet unknown mechanisms, and uncouples the gap junctions (by phosphorylation of connexin-43) via mitogen activated protein kinase (MAPK) [139, 141, 142]. Reduced production and entrance of cGMP into the oocyte in turn increases PDE3A which ultimately hydrolyses cAMP and enables resumption of meiosis.

Luteal Dynamics

Based on the status of the CL, the estrous cycle may be divided into four successive phases: (i) proestrus, when the CL is regressing and an ovulatory follicle develops; (ii) estrus, the absence of a functional CL and the period of sexual receptivity when final follicular maturation and ovulation occur; (iii) metestrus, the period of CL formation; and (iv) diestrus, when the CL is mature and actively produces progesterone [143]. Proestrus and estrus have been referred to as the follicular phase, while metestrus and diestrus are the luteal phase, though "luteal phase" should not be taken to imply the lack of follicle development. To the contrary, follicular waves continue to develop throughout follicular and luteal phases of the estrous cycle, as described above.

Origin of the CL

The growth and demise of the CL during the estrous cycle represents one of the most rapid dynamic processes in the body. At the time of ovulation, antral contents are evacuated and the wall of the ovulatory follicle collapses into undulations within the vacated antrum (Figure 25.15). The granulosa and theca cells of the former follicle then expand beyond the volume of the former follicular antrum through a dramatic process of vascularization and luteinization to form a functional CL. This temporary steroid-producing gland undergoes marked structural and functional changes in a short time-span and has been the subject of extensive morphological studies (Figure 25.15) (reviewed in [144]). Plasma progesterone concentration is highly correlated with CL weight, volume [145], histomorphology [146], and ultrasound morphology [144].

During metestrus, blood vascular components occupy the greatest percentage volume of the CL. The luteal cells undergo hypertrophy at mid-diestrus, during peak progesterone production, in association with their greatest volume density. An increase in luteal cell size during the formation phase and decrease during the regression phase

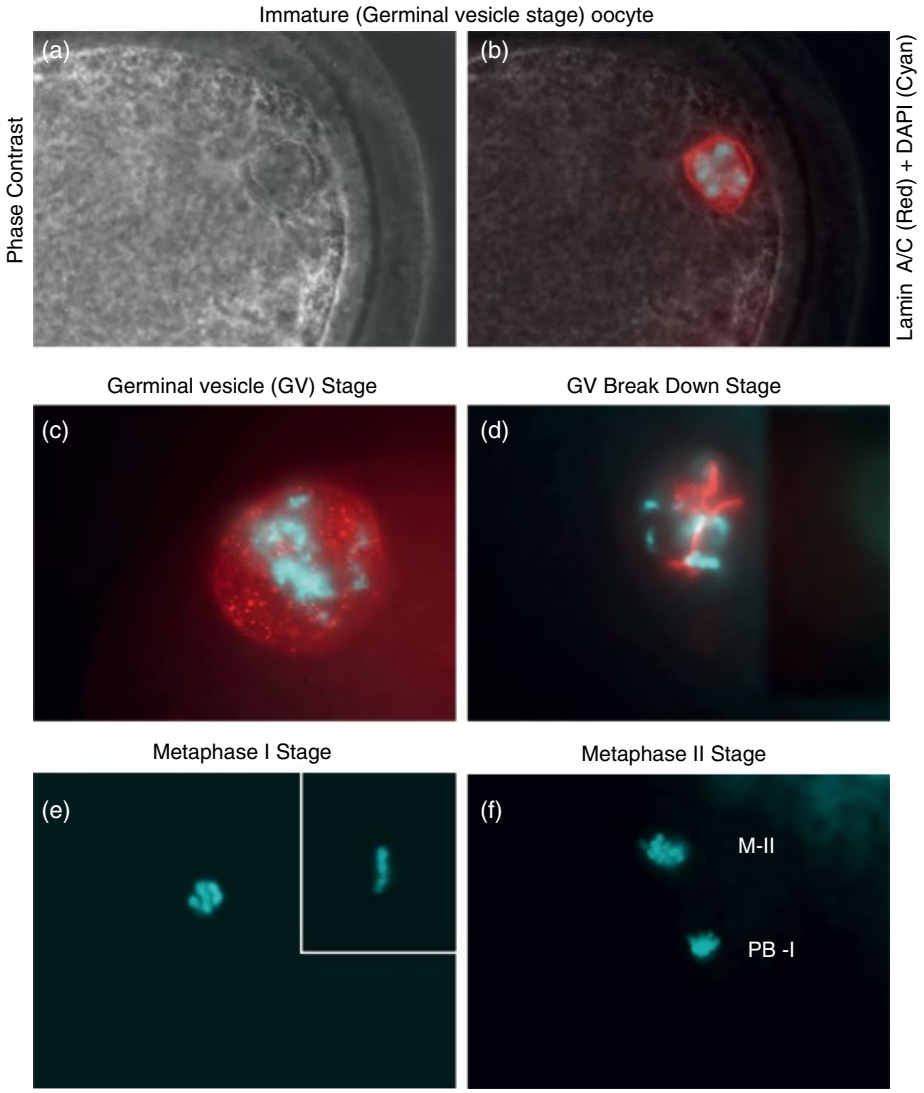


Figure 25.14 Stages of nuclear maturation of bovine oocytes. (a) Phase-contrast image of an immature oocyte with an intact nuclear membrane (germinal vesicle; GV stage) obtained from a 4-mm follicle. (b) Anti-lamin A/C (stains nuclear membrane red) and DAPI (stains DNA cyan) stained images merged with the phase-contrast image in (a). (c) Nucleus of an immature oocyte with an intact nuclear membrane (GV stage). (d) Maturing oocyte showing germinal vesicle breakdown (GVBD stage). (e) Metaphase I (MI); inset shows MI plate at a right-angle. (f) Metaphase II (MII) and the first polar body (PB-I). *Source:* Adapted from [130].

parallel progesterone secretion. During proestrus, the regressing CL decreases in weight, in volume densities of luteal cells and blood vascular components, and in progesterone production. There is a concurrent increase in connective tissue and hyalinization of blood vessels. Individual luteal cells undergo degenerative changes reflected by decreased cell size and accumulation of coarse lipid droplets in the cytoplasm. These morphological changes are associated with progressive changes in plasma progesterone concentration from <2 ng/ml at three days after ovulation to about 4 ng/ml by six days and peak concentrations >6 ng/ml between 10 and 14 days postovulation, followed by decreasing levels after 16 days due to luteolysis [83].

Ultrasonographic Detection and Morphology of the CL

The reliability and accuracy of ultrasonography for detecting the CL and the relationship between ultrasound image attributes and CL function have important implications in clinical settings for assessing normalcy and stage of the cycle. In a comparison between ultrasonographic detection versus gross anatomic dissection, there was 83% agreement in inexperienced operators and 97% agreement in experienced operators [147]. The growing CL was detected by day 2 (day 0 = ovulation) in >95% of 80 estrous cycles, and the regressing CL was detected, on average, 1.4 days into the next estrous cycle [147].

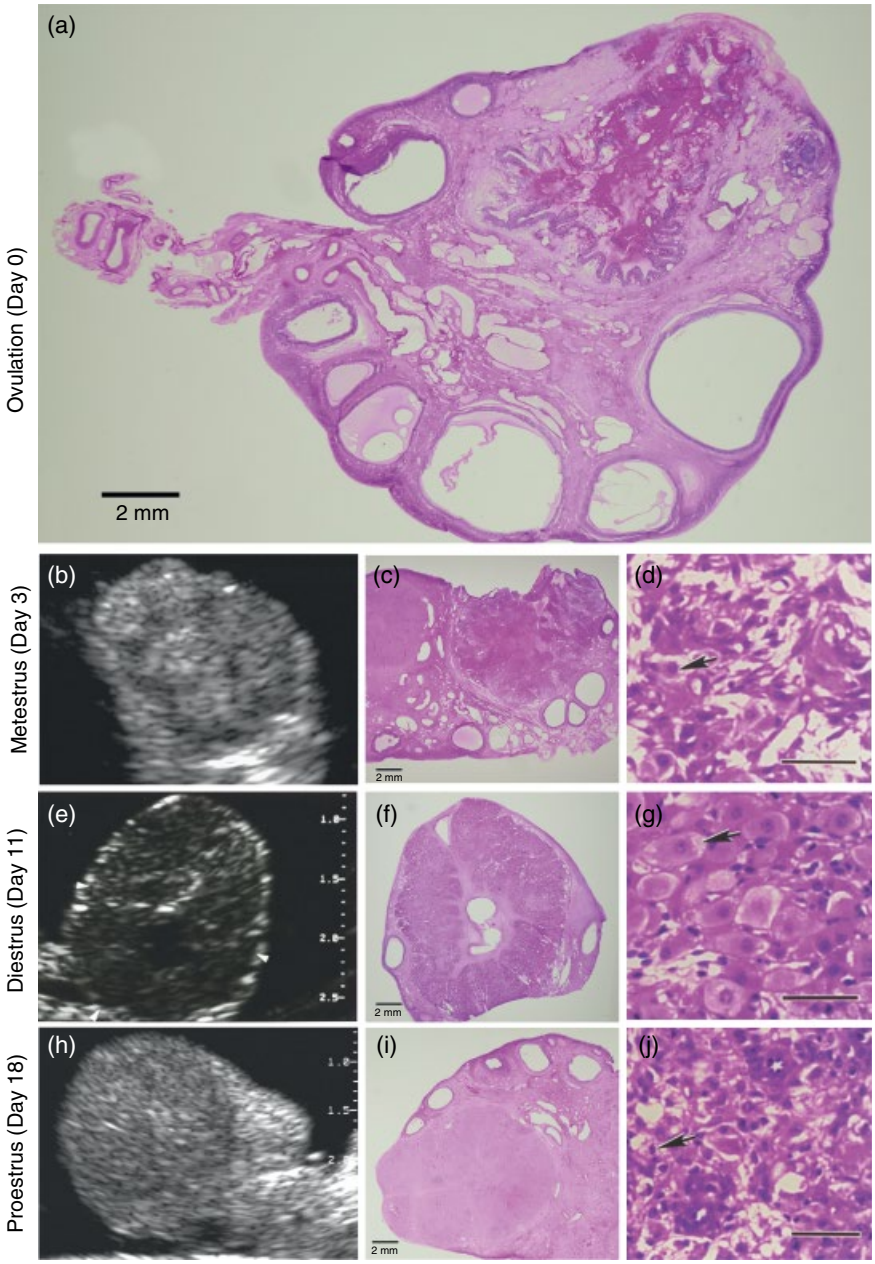


Figure 25.15 Development of the corpus luteum in cattle. (a) Histological section of an ovary taken within one hour of ovulation. Note the collapsed follicle with extensive crenulation of the follicle wall (arrowhead) and blood clot in the antrum. Note also numerous small antral follicles in the ovary, typical of new wave emergence at the time of ovulation. Ultrasound morphology (b, e, h), low magnification histology (c, f, i), and tissue details (d, g, j) of bovine corpora lutea during metestrus (b, c, d; day 3 after ovulation), mid-diestrus (e, f, g; day 11 after ovulation), and proestrus (h, i, j; day 18 after ovulation). Arrows indicate luteal cells. Greater angiogenesis during metestrus and hyalinization of blood vessels during proestrus (luteal regression) resulted in brighter echotexture of ultrasound images at these stages compared to diestrus. Histological images were taken from 5-μm-thick paraffin sections stained with hematoxylin and eosin. Scale on the ultrasound images (b, d, f, h) is in centimeters. Scale bar is 2 mm for a, c, f, i and 10 μm for d, g, j.

A large proportion (40–70%) of normal corpora lutea have a central fluid-filled cavity during their development; the remainder are solid structures (reviewed in [144, 148]). The cavity of the CL is filled with a serous transudate and remains anechoic during its entire existence, as opposed to the organized clot and appearance of a network of echogenic lines within the central cavity of the equine CL. The central cavity becomes smaller and often disappears as the CL matures. Plasma progesterone concentrations are not different between CL with or without a central cavity. The profile of luteal tissue area (minus area of a cavity if present) detected by ultrasound paralleled the profile of plasma progesterone concentration, with the exception that during luteolysis progesterone

began to decrease one to two days before ultrasonographic regression was apparent [62].

Changes in ultrasound image attributes (echotexture) of the CL are also reflective of CL function in cattle (Figure 25.15) [144]. Higher pixel values, obtained by computer-assisted quantitative echotexture analysis during luteal gland formation (metestrus) and regression (proestrus group) were similar to the pattern observed in horse and pony mares by subjective scoring of echotexture [149] and pixel analysis [150, 151] of corpora lutea recorded *in vivo;* by transrectal ultrasonography. In a test of the hypothesis that ultrasound images may be digitally "stained," computer algorithms were developed to produce a "virtual histology" through subtle differences in echotexture resulting from different ovarian structures [152]. Quantitative analysis showed that the "sticks" algorithms increased the statistical differences in the echotexture of ovarian stroma versus the CL, representing a first step toward virtual histology of ultrasound images and understanding dynamic changes in the ovary at the microscopic level in a safe, repeatable, and non-invasive way [153].

Vascular Flow Dynamics in the CL

Major changes in the microvasculature of the ovary occur during growth and regression of large follicles and the CL [144, 154, 155]. Three variations of the Doppler technique have been used to study vascular flow: (i) spectral Doppler is used to display vascular flow over time in a waveform; (ii) color-flow Doppler permits superimposition of information about vascular flow direction on the B-mode gray-scale image; and (iii) power-flow Doppler is used to superimpose low velocity vascular flow information (without directional information) on the B-mode gray-scale image. Doppler studies have provided visual evidence for time-related changes in blood flow within the preovulatory follicle wall of cows in relation to LH surge [154, 156]. Initial results indicate that a biphasic blood flow response to LH may occur in the bovine preovulatory follicle. An initial increase in blood flow was accompanied by serration of the follicular granulosa layer within 3 hours of LH treatment and a second increase in blood flow was detected around 20 hours after LH [156]. Distinct changes in vascular flow patterns during luteogenesis, luteolysis, and early pregnancy and in response to GnRH, prostaglandin F2α (PGF), PGE, PGI, oxytocin, and NOS have been reported in cattle [154, 157–161].

Luteolysis

In the early 1970s, PGF was found to be the natural luteolysin in cattle [162, 163], and since that time PGF has become the most commonly used treatment for elective induction and synchronization of estrus in cattle [164–166]. Pulsatile release of PGF from the uterus initiates luteolysis beginning four to six days before ovulation [167, 168]. Early studies showed that the maturity of the CL at the time of PGF treatment influenced the luteolytic response [169]; PGF did not effectively induce luteolysis during the first five or six days following estrus. Lack of responsiveness was thought to be a result of a lack of PGF receptors in immature CL, but more recent work has refuted this theory by demonstrating the presence of PGF receptors in CL as early as two days after ovulation [170]. The reason for the resilience of the immature CL to PGF remains unclear, but some suggest that the mature CL may possess a positive feedback loop involving luteal oxytocin and tumor necrosis factor which elicits endometrial release of PGF and sustains the process of luteolysis [171, 172]. If this notion is correct and immature CL do not possess the positive feedback loop, then multiple injections of PGF may be expected to complete the luteolytic process in early stage CL. In this regard, luteolysis and ovulation was induced in 56/60 heifers (93%) given PGF in the morning and evening of either the fourth or fifth day after estrus in two studies involving ovarian superstimulation [173, 175]. Recent studies show a distinct cause-and-effect balance between the luteotrophic effect of endogenous LH pulses and the luteolytic effect of pulses of PGF; repeated PGF pulses from the uterus ultimately overcome the resuscitating effects of LH to complete luteolysis (reviewed in [174]).

Ovarian Dynamics During Different Reproductive States

Pre- and Peripubertal Periods

In the only study on ovarian follicle dynamics during the pre- and peripubertal periods, transrectal ultrasonography was used to monitor daily changes in follicle development in calves as young as two weeks of age; calves were monitored at regular intervals during the first year of life [175–177]. Puberty was defined as the time of the first ovulation and was determined to be 52–56 weeks (12–13 months) in the Hereford-cross heifers used in the study. Remarkably, follicular development occurred in a wave-like fashion, similar to that in adults, at all ages examined. Individual follicle development was characterized by growing, static, and regressing phases, and periodic surges in serum concentrations of FSH were associated with each follicular wave emergence (Figure 25.8). FSH surges spanned a mean of three days and were maximal one day before wave emergence. It was concluded that the mechanisms controlling the well-ordered phenomena of wave emergence, follicle selection, and follicle regression in mature cattle were also extant in prepubertal heifers.

The diameter profile of the dominant as well as the largest subordinate follicle in prepubertal calves increased

with age (Figure 25.16). The rate of increase was greatest from 2 to 8 weeks of age and again between 24 and 40 weeks of age, in temporal association with increases in mean concentrations of LH [176]. The number of follicles detected (≥3 mm in diameter) also increased dramatically between 8 and 14 weeks of age, and then appeared to decrease as puberty approached, in temporal association with the early rise and subsequent tapering of mean FSH concentrations. The developmental reason for the early rise in circulating concentrations of LH and FSH between 4 and 14 weeks of age is not clear, but it may reflect initial maturation of the hypothalamo-pituitary axis and subsequent sensitivity to negative feedback by ovarian steroids. This notion is supported by data illustrated in Figure 25.16 and in observed changes in the interval between the emergence of dominant follicles of successive waves (interwave interval). The interwave interval was relatively long (8–9 days) at 2 and 8 weeks of age, but was significantly shorter (6.8 days) at 14 weeks. The interwave interval gradually increased thereafter until puberty (7–9 days).

Examination of data from the period immediately preceding first ovulation and encompassing the subsequent two interovulatory intervals revealed unique characteristics at the onset of ovulatory cyclicity in cattle [177]. The first ovulatory cycle was short (7.7 ± 0.2 days) and the first ovulation occurred after the dominant follicle had entered the static phase; that is, the dominant follicle was older at the time of ovulation than its counterpart of later cycles. The first CL (from the aged ovulatory follicle) was smaller and shorter-lived than corpora lutea of subsequent cycles, thus resulting in an abbreviated interovulatory interval. The second interovulatory interval was of normal duration (20.3 ± 0.5 days) and was composed of two or three follicular waves. Mean serum estradiol and LH concentrations, and LH pulse-frequency increased as first ovulation approached, but FSH concentrations did not. Surges in FSH were associated with follicular wave emergence, as seen previously. These results demonstrate a striking similarity between the onset of puberty in young heifers and recrudescence of ovulatory cyclicity in postpartum cows (see below).

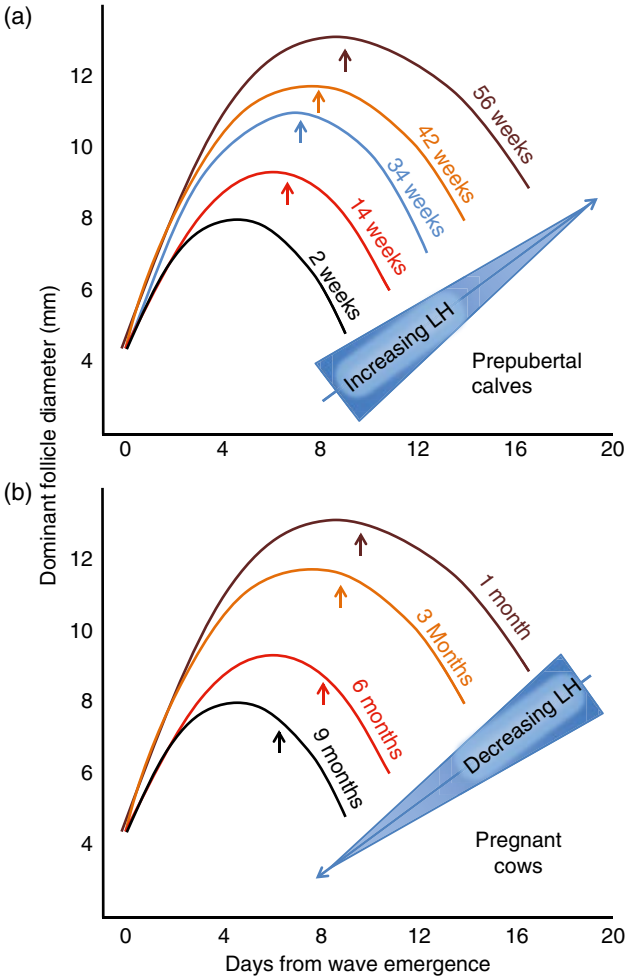


Figure 25.16 Diameter profiles of the dominant follicle of follicular waves during prepubertal development in calves (a) and during gestation in pregnant cows (b) as detected by daily transrectal ultrasonography. (a) The profile represented at 56 weeks of age is of the wave immediately preceding puberty (first ovulation). From 14 to 56 weeks of age, the interwave interval (indicated by arrows) increased from 6.8 to 9.2 days. (b) In pregnant cows from 1 to 9 months of gestation, the interwave interval (indicated by arrows) decreased from 9.6 to 6.4 days. *Source:* Data from [43, 57, 178]. Changes in dominant follicle profiles are associated with changes in circulating concentrations of LH.

Follicular Development During Pregnancy

Results of three separate studies are consistent in that the wave phenomenon occurs throughout pregnancy, except for the last 21 days when no follicles ≥6 mm were detected [43, 57, 178]. In the presence of progesterone [43] (endogenous or exogenous), anovulatory follicular waves emerge at regular intervals. However, the maximum diameter of the dominant follicle of successive waves decreased and was associated with a successive decrease in the interwave interval (i.e. the period of dominance became shorter; Figure 25.16). An initial decrease occurred immediately after the first follicular wave of pregnancy, and a subsequent sharp decrease occurred after the fourth month of pregnancy. Progesterone suppresses growth of the dominant follicle in a dose-dependent manner [83], and the progressive decrease in follicular dominance seen in pregnant cattle was attributed to the suppressive effects on LH of rising progesterone levels during mid-pregnancy and rising estrogen levels near term [178].

As in non-pregnant cows, periodic surges of FSH were associated with periodic emergence of follicular waves in

pregnant cows. In addition, the magnitude and frequency of FSH surges were influenced by the size of the dominant follicle. Waves in which the dominant follicle reached a diameter of ≥10 mm were associated with longer intervals between successive FSH surges and lower concentrations of FSH. Conversely, waves with smaller dominant follicles (i.e. 6–9 mm) were associated with shorter intervals and higher concentrations of FSH. A similar interrelationship between FSH and follicular wave dynamics was observed in prepubertal calves.

Follicular Development During the Postpartum Period

In all species in which it has been studied, lactation and suckling have been shown to have a suppressive effect on ovarian follicular development. The degree of suppression in cattle has been associated primarily with energy balance (nutritional status, body condition, milk production, parity) and periparturient disorders (metritis, endometritis, mastitis, lameness) (reviewed in [179–181]). The interval from calving to first ovulation in dairy cows was a mean of 21 days [182] (range 10–55 days), but was significantly longer in primiparous cows than multiparous cows [183] (31.8 ± 8.3 days vs 17.3 ± 6.3 days). Emergence of the first follicular wave postpartum ranged from 2 to 7 days (mean 4.0 days) after calving in primiparous Holsteins [178], and the dominant follicle of the first postpartum wave was ovulated in 14/19 multiparous Friesian cows (74%) [184] on mean day 27 (range 12–58) [185]. Recrudescence of follicular waves was observed within 10 days of calving in beef cattle [186], but the first ovulation occurred somewhat later than in dairy cattle (mean 30.6 days) and only rarely from the dominant follicle of the first postpartum wave (11%) [186].

The first postpartum ovulation is frequently associated with an absence of estrous behavior and is often followed by a luteal phase of short duration (reviewed in [187]). The first ovulation was not accompanied by estrus in 17/18 (94%) postpartum dairy cows [184], and the length of the first postpartum interovulatory interval varied depending on when the first follicle destined to ovulate emerged. The first postpartum interovulatory interval was short (mean 11.2 days) in approximately 25% of dairy cows, was of normal duration (mean 20.6 days) in another 25%, and was long (mean 30.0 days) in the remaining 50% of cows [184]. Short cycles were associated with later detection of the first follicle destined to ovulate (i.e. ≥10 days postpartum), whereas normal and long cycles were associated with earlier detection of the first follicle destined to ovulate (i.e. usually ≤10 days). These findings are remarkably consistent with those of another study [182] in which short postpartum anovulatory periods (around 14 days) were followed by a normal-length cycle (18–21 days), and longer postpartum anovulatory periods (21–25 days) were followed by a short cycle (<14 days). In the majority of beef cows (78%), ovulation occurred from the second, third, or fourth postpartum follicular wave, and as in dairy cattle, the first ovulations occurring after 20 days (16/18 cows) were followed by a short cycle (14/16 cows) [186]. Short cycles were associated with a short luteal phase, a smaller CL, and lower circulating progesterone concentrations. The short luteal phase following the first postpartum ovulation has been attributed to premature release of PGF2α by the uterus as a result of lower numbers of progesterone receptors and greater numbers of oxytocin receptors in endometrial cells (reviewed in [187]); however, luteal inadequacy as a result of altered preovulatory granulosa/theca cell differentiation in a low LH environment remains to be tested.

Follicular Development During Old Age

Decreased fertility with maternal aging has been well documented in cattle and humans [188–190]. Endocrine and ovarian characteristics of reproductive aging were characterized in a series of studies in which old cows (≥15 years) were compared with their young daughters (≤5 year) [191–193]. Mean circulating FSH concentrations were consistently higher in old cows than in their daughters, but the expected pattern of FSH secretion and wave emergence was maintained in old cows, i.e. each ovarian follicular wave was preceded by a surge in circulating FSH. Despite elevated FSH, fewer 4- to 5-mm follicles were recruited into each follicular wave in old cows than in their daughters. This interesting inverse relationship between the number of follicles recruited into a wave and the peak concentrations of FSH has also been reported in studies documenting the repeatability of follicle numbers within individuals [70, 118]. The duration of the interovulatory and interwave intervals did not change with age, but the ovulatory follicle of old cows with a two-wave pattern was smaller at the time of ovulation than that of young cows. Similarly, the diameter of the CL was smaller and the plasma concentration of progesterone tended to be lower in old versus young cows. There was no age effect on circulating LH concentrations or LH pulse frequency.

The responsiveness of the hypothalamo-pituitary axis in young and old cows was examined in a study involving estradiol/progesterone-based ovarian synchronization [192]. Steroid treatment suppressed circulating FSH in both age groups, and the intervals from treatment to subsequent FSH peak and wave emergence were not different between old and young cows. In a study of the ovarian response to superstimulatory treatment [194], fewer small (<5 mm) follicles were recruited into the follicular wave

and fewer follicles in all size categories developed after ovarian superstimulation in old cows than in their young daughters. On average, the young daughters had eight more ovulations per cow than their mothers. Lastly, data from a study in which embryos from old cows and their daughters were transferred to age-matched recipient cows [191] revealed that the oocytes from old cows were markedly less competent and produced only half as many embryos as their daughters.

Conclusions and Implications

Knowledge of normal follicular and luteal dynamics and the mechanisms controlling these dynamics have had a direct impact on clinical and breeding management practices in cattle. The concepts represented in Figure 25.9 have been used as a basis for developing protocols for ovarian synchronization for fixed-time insemination and for ovarian superstimulation for the purposes of *in vivo;* and *in vitro;* embryo production and embryo transfer, and for controlled breeding management programs in dairy and beef herds. The concepts of the wave pattern of follicle development and selection imply predictable variations in the number and maturity of specific classes of follicles (e.g. growing, static or regressing, dominant, and subordinate follicles) that may or may not be capable of responding to certain treatments. Importantly, the dynamics of follicular development are overlain by distinct influences of the CL. Some examples of the impact of endogenous ovarian rhythms on practice are offered below.

Synchronization

In cows in which luteolysis is effectively induced by PGF treatment, the ensuing estrus is distributed over a six-day period [195]. The most consistent response (i.e. the least variability in interval to estrus) resulted from PGF treatment during early diestrus (7 and 8 days after estrus) and later diestrus (15 days after estrus) than from treatment during mid-diestrus (10–13 days after estrus) [195]. Subsequent studies have documented that much of this variation is due to follicular status at the time of treatment. Treatment when the dominant follicle of a wave is in its late growing (≥9 mm) or early static phase will result in ovulation of that follicle within two to three days, whereas treatment after the mid- to late-static phase (i.e. when it is no longer viable) will result in ovulation of the dominant follicle of the next wave four to five days after treatment. That is, if PGF is given at the end of the period of functional dominance of one follicle, several days are required for a new dominant follicle to grow sufficiently to ovulate [167, 196, 197].

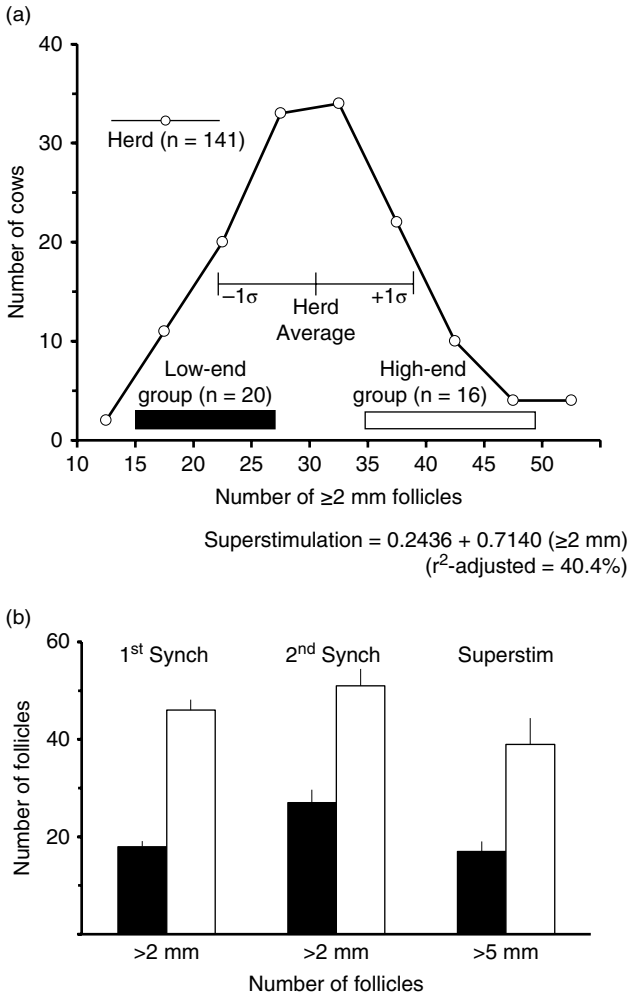


Figure 25.17 Frequency distribution of Hereford-cross beef cows in a herd (n = 141, line) based on the number of follicles detected in both ovaries by ultrasonography at the time of follicular wave emergence. (a) Based on antral follicle count, a sample of cows from the low end (n = 20, black bar) and high end (n = 16, white bar) were selected for successive synchronization and superstimulation to determine the repeatability and impact of antral follicle count. (b) The number of follicles detected at emergence of the first and second synchronized waves, and after ovarian superstimulatory treatment in the low-end group (black bars) and high-end group (white bars). The number of follicles detected was significantly greater in the high-end group after both synchronizations and after superstimulation. The superstimulatory response was related in a simple linear fashion to the number of follicles detected at wave emergence [superstimulatory response = 0.2436 + 0.7140 (# of follicles ⩾2 mm); r^2-adjusted = 40.4%]. *Source:* Adapted from [118].

Antral Follicle Count

The number of follicles detectable by ultrasonography varies widely among cows and according to follicular wave status [44, 70, 112, 118]. However, differences in follicle numbers are not associated with wave sequence (i.e. similar between wave 1, wave 2, and wave 3) or the number of

waves per estrous cycle (i.e. two- and three-wave patterns; reviewed in [44]). Although individuals vary greatly, the intrinsic number of follicles at wave emergence is highly repeatable within individuals [70, 118]. In a direct comparison of high-follicle count vs low-follicle count cows [118], the number of follicles recruited into a wave was highly correlated with the number of follicles recruited into successive waves, and the superstimulatory response was highly correlated with the number of follicles recruited into a follicular wave for a given individual (Figure 25.17). This characteristic now forms the basis of a simple ultrasound test to predict the superstimulatory response in cattle.

In a recent study of the effect of restricting maternal nutrition to 60% of maintenance requirements during the first third of gestation, calves born to nutritionally restricted mothers had 60% lower antral follicle counts compared to calves born to mothers fed control diets [198]. Furthermore, results suggest that fertility may be compromised in animals with low antral follicle counts due to effects on oocytes, progesterone, and the endometrium compared to animals with high antral follicle counts; postpartum dairy cows with high antral follicle counts had higher pregnancy rates and shorter calving to conception intervals, and received fewer services during the breeding season compared to cows with low counts [198].

Ovarian Superstimulation

The greatest sources of variability in response to ovarian stimulation are the status of the follicular wave at the time stimulatory treatment is initiated and the intrinsic number of follicles present at the time of wave emergence in a given individual. Results of studies in cattle have documented that the superstimulatory response is (i) inferior when treatment is initiated after selection of the dominant follicle; (ii) greater when treatment is initiated at the time of wave emergence rather than one or two days later; and (iii) similar between wave 1 (rising progesterone) and wave 2 (high-endogenous progesterone (reviewed in [118]). Only approximately 20% (four or five days) of the estrous cycle is available for initiating treatment at the time of follicular wave emergence. Therefore the majority (80%) of the estrous cycle is not conducive to an optimal superovulatory response. The superstimulatory response may be predicted to be approximately 70% of the number of follicles ≥3 mm at the time of wave emergence [118]. As a result of these findings, superstimulatory protocols in cattle now incorporate synchronization of follicular wave emergence.

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Maternal Recognition and Physiology of Pregnancy

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Introduction

Maternal recognition of pregnancy encompasses the process whereby a biochemical signal is generated to prevent luteal regression, allowing the corpus luteum to persist and continue to secrete adequate progesterone. The requirement of progesterone for the successful maintenance of pregnancy exceeds the length of the bovine estrous cycle and therefore the pregnant female must quickly recognize that a conceptus is present. In cattle, this signal involves conceptus secretion of interferon (IFN)-tau which further stimulates luteotropic and antiluteolytic signals. Although some studies use these words interchangeably, we will further define a luteotropin as a signal that stimulates luteal secretion of progesterone, while antiluteolytic substances act as a signal to block luteolysis by inhibiting or masking endogenous luteolytic signals. This maternal recognition of pregnancy signal is also occurring well in advance of conceptus implantation, which begins during the third week of pregnancy and is classified as a superficial attachment in cattle.

Several biological assays have focused on elucidating substances involved in prolonging the lifespan of the bovine corpus luteum. The majority of these earlier assays focused on examining estrous cycle length in non-pregnant cattle injected with pituitary extracts. In 1965, researchers at Cornell University reported that luteinizing hormone (LH) was the luteotropin for cattle and, indeed, injections of pituitary extracts or purified bovine LH extended the bovine estrous cycle by 11 or 16 days, respectively [1]. This study confirmed the luteotropic actions of LH, which could serve as the endogenous biochemical signal from the conceptus; however, apart from primates and mares, no other mammals appear to possess both genetic subunits for a chorionic gonadotropin. Therefore a chorionic gonadotropin possessing LH properties is not the signal for maternal recognition of pregnancy in cattle. However, these early studies did contribute to the foundational groundwork examining mammalian species differences in prolonging the lifespan of the corpus luteum, which is an important component of the conceptus–maternal cross-talk during maternal recognition of pregnancy.

These early results led researchers to examine other potential substances originating from the conceptus that may act in a luteotropic and/or antiluteolytic nature to prevent luteolysis during pulsatile release of prostaglandin (PG)$F_{2\alpha}$. Intrauterine infusions of estradiol-17β and prostaglandin E_2 (PGE_2) prolong the lifespan of the bovine corpus luteum as well as progesterone secretion by five to six days compared with non-pregnant controls [2]. The luteotropic actions of estradiol-17β are more than likely indirect because estrogens have been shown to inhibit LH-induced secretion of progesterone by cultured bovine luteal cells [3]. The indirect actions of intrauterine estradiol-17β on prolonging luteal lifespan may involve an increase in uterine and/or ovarian blood flow. A local increase in blood flow to the gravid uterine horn occurs coincidental with maternal recognition of pregnancy in the sow, ewe, and cow [4]. In addition, this local increase in uterine blood flow may help to redirect prostaglandin transport from the uterine horn to the adjacent ovary via the utero-ovarian plexus. PGE_2 directly stimulates progesterone secretion and cyclic adenosine monophosphate (AMP) production of bovine luteal cells *in vitro* and in a similar manner to that of LH [5]. Moreover, both the bovine endometrium and corpus luteum express the complete prostaglandin biosynthesis pathway and the differential expression of these components during the critical period of maternal recognition of pregnancy (days 15–17 of the estrous cycle) and can exert luteotropic or antiluteolytic actions [6].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Interferon (IFN)-tau, first identified as bovine trophoblast protein 1 [7], is recognized as the maternal recognition of pregnancy signal in cattle [8]. IFN-tau secretion by embryonic trophoblast cells is highest between days 15 and 17 and is observed up to day 28 of pregnancy; an inadequate response of the endometrium to IFN-tau is likely to be one of the major reasons for improper production and action of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and PGE_2 leading to pregnancy failure. *in vitro* cultures of bovine endometrial cells with increasing concentrations of IFN-tau show preferential production of PGE_2 compared with $PGF_{2\alpha}$ [9]. Moreover, the spatial and temporal relationship of prostaglandin metabolism, transport, and receptor density changes substantially in the uterine endometrium and myometrium following IFN-tau exposure [6]. Elevated physiological concentrations of IFN-tau from the conceptus will selectively increase the ratio of PGE_2 to $PGF_{2\alpha}$. This relationship sets up an antiluteolytic effect of IFN-tau combined with a luteotropic effect of PGE_2 which eventually leads to prolonged corpus luteum lifespan and proper establishment of pregnancy. In addition, embryonic secretion of PGE_2 may produce a synergistic effect with the increased endometrial production of PGE_2 that was stimulated by conceptus secretion of IFN-tau. This PGE_2 can be competitively transported through the utero-ovarian plexus to the ovary, where it increases production of luteal PGE_2, thereby favoring luteal maintenance as a luteotropin [6]. In contrast, an elevated ratio of $PGF_{2\alpha}$ to PGE_2 will stimulate uterine contractility and competitive transport of $PGF_{2\alpha}$ through the utero-ovarian plexus toward the corpus luteum, thereby initiating luteal regression. Together, uterine contractility and luteal regression can lead to pregnancy failure and/or a return to a new estrous cycle following a normal lifespan of the corpus luteum.

Prevention of corpus luteum regression and continual secretion of adequate progesterone is vital for successful maintenance of pregnancy to term. The primary targets for progesterone are the reproductive tract. The amplitude of progesterone secretion can influence the early uterine environment, including the inhibition of mitotic division of the endometrium, the induction of stromal differentiation, and the stimulation of additional glandular secretions of the uterine epithelium or histotroph [10]. This increase in glandular uterine secretions of nutrients and growth factors is essential for early conceptus development [11]. In 1956, researchers proposed the progesterone block hypothesis, which states that progesterone maintains pregnancy by directly blocking labor, and removal of the progesterone source will initiate parturition [12]. Later studies revealed that progesterone supplementation prevented the insertion of gap junction proteins between uterine smooth muscle cells by decreasing the expression of connexin-43 [13]. Progesterone has also been shown to downregulate uterine contractility-associated genes [14]. In general, cows with less than 0.5 ng/ml of progesterone have elevated contractility in circular smooth muscle cells of the myometrium compared with that in diestrus cows (>4.0 ng/ml progesterone) [15]. These differences in myometrial excitation–contraction are brought about by the genomic actions of progesterone, which lead to a relaxed and quiescent uterus able to maintain a successful pregnancy until progesterone withdrawal [14]. In addition, progesterone synthesis depends on luteal production for the first 200 days of gestation; however, the extragonadal source of progesterone, primarily the bovine placenta, does not secret adequate amounts of progesterone until the third trimester of pregnancy. This placental progesterone secretion is dependent on luteal ovarian input. The luteal placental shift in progesterone secretion, which may contribute to the maintenance of pregnancy, only occurs after $PGF_{2\alpha}$-induced luteolysis or in ovariectomized animals [16]. Moreover, in the cow, the corpus luteum may exert control over placental steroidogenesis, which does not take over progesterone secretion until ovarian luteal deficiencies become apparent during late gestation.

Conceptus Elongation and Extraembryonic Membranes

The early elongation of the bovine conceptus involves exponential increases in length and width of the trophectoderm and requires differentiation of extraembryonic membranes as well as maternal endometrial influences. This elongation begins on day 14, following the hatching of the ovoid blastocyst at approximately day 9–10, and forms a filamentous conceptus by day 19–20 after insemination [17]. On day 14, the majority of ovoid or tubular embryos of approximately 5 mm in length will start to form an epiblast, defining the embryonic disk, which is discernible from the epithelium of the trophoblast. Both of these compartments are internally lined by the continuous hypoblast, of which the majority stains positive for α-fetoprotein [18]; however, the histological appearance of the hypoblast varies in relation to its spatial orientation to the epiblast versus the trophoblast cell layers. The spatial orientation results in two types of hypoblast cells: squamous cell types if located next to the trophoblast layer, or cuboidal to columnar cell types underneath the epiblast or embryonic disk [18]. At this point a distinctive basal lamina is formed between the trophoblast or epiblast and the hypoblast cell layers. In addition, adjacent epiblast cells are connected via tight junctions, as well as peripheral epiblast cells next to the trophoblast cells. Presumptive signs of early embryonic mortality in the tubular conceptus consist of apoptotic epiblast cells or a loosening of the hypoblast

from the trophoblast. During this elongation phase the initial folds of the amnion will form as an outgrowth of the trophoblast cells adjacent to the epiblast, while the hypoblast is not included in the formation of this extra-embryonic membrane. The most discernible difference during this time-frame is the hypertrophic and hyperplastic trophectoderm growth of the differentiating chorionic sac, which may grow from a few centimeters up to 30 cm or more [19].

The early attachment period of days 20–33 post insemination proceeds with sequential stages of precontact, apposition, and adhesion between the uterus and the chorion, and ends in a microvillous interdigitation of the uterine epithelium and trophoblast. This time period coincides with several reports of limited success in flushing intact conceptuses for experimental use [20]. During the early attachment phase the development of the allantois begins, and on day 21 after insemination a strong positive association between embryo length and width of the allantois can be observed [18]. Some researchers have observed abnormalities or underdevelopment of the extra-embryonic membranes following *in vitro* fertilization or somatic cell nuclear transfer; however, the underlying mechanisms for development failure are not clearly understood. Therefore embryonic mortality during these time points may be caused by a difficulty in nutrient mobilization brought about by the allantois showing deficient vascularization, growth retardation, or its absence [21]. In relation to the dramatic elongation of the trophectoderm of the chorionic sac, the allantois undergoes gradual growth from days 21 to 25 post insemination, when it will start to occupy both uterine horns and begin fixation to the chorion to form the chorioallantois placenta; however, this fusion remains incomplete until days 60–70 of pregnancy [21].

In addition to the morphological change from a spherical conceptus into a filament-like tube during conceptus elongation, several molecular markers have been characterized in relation to growth and cellular differentiation. The trophoblast mononucleate cells of the trophectoderm will begin to proliferate and differentiate, which may be induced by specific transcription factors such as POU-domain class 5 transcription factor, ERG, and CDX2 [22]. These transcription factors have been shown to regulate genes such as IFN-tau and trophoblast domain proteins, which are upregulated during maternal recognition of pregnancy. In addition, the differentiation of trophoblast mononucleate cells into trophoblast giant cells coincides with an increase in pregnancy-associated glycoproteins, prolactin-related proteins, and placental lactogen. These proteins are associated with cellular differentiation of the trophectoderm; however, they may not be associated with the proliferation of the trophectoderm during conceptus elongation into the filament-like tube [22]. Transcriptome profiling using a bovine-specific microarray has revealed the upregulation of approximately 500 genes and the downregulation of only 26 genes during transition from a blastocyst (day 7) to an ovoid tubular conceptus (day 14). Of these genes, IFN-tau was positively correlated with conceptus size; therefore it may be a key factor in trophectoderm proliferation during the elongation phase. After further elongation of the bovine conceptus (gestational days 17–19), specific genes involved in trophoblast cell differentiation, migration, and adhesion are upregulated, all of which play a role in fusion of the trophoblast cells to the endometrium of the uterus. Of the 80 genes upregulated during this phase, the *CD9* gene, which encodes for cell-surface glycoprotein belonging to the tetraspanin family, of the differentiated trophoblast giant cells more than likely plays a role in cellular migration and adhesion of the conceptus to the maternal endometrium during early attachment (gestational day 20).

Uterine Histotroph

The growth, development, and welfare of the bovine conceptus depend on uterine histotrophic nutrition derived from the secretions of uterine endometrial glands. The uterine endometrium is a complex tissue composed of a luminal epithelial cell layer, superficial glandular epithelium, and deep glandular epithelium, as well as stromal cells with a fibroblast-like morphology. In addition to autonomous regulation of trophectoderm proliferation and differentiation during conceptus elongation, uterine input via endometrial secretions will drive early conceptus development. As previously mentioned, the bovine conceptus must secrete sufficient quantities of IFN-tau by day 16 of pregnancy in order to inhibit the production of luteolytic pulses of $PGF_{2\alpha}$ by the endometrium to allow for maternal recognition of pregnancy. In addition to conceptus secretions that alter endometrial pathways in a paracrine manner, ovarian hormones have been shown to stimulate or inhibit endometrial secretions and glandular output. A number of studies have demonstrated that peripheral concentrations of progesterone during the early postconception period are associated with advanced conceptus elongation and higher pregnancy rates in cattle and sheep. In contrast, lower concentrations of progesterone in dairy cows are associated with decreased conceptus development. Ovarian progesterone can influence the early uterine environment, including endometrial sections of nutrients and growth factors that are essential for early conceptus development. Moreover, supplementing exogenous progesterone from day 5 to 9 after insemination resulted in a fourfold increase in trophoblast length and a sixfold increase in

uterine concentrations of IFN-tau [23]. In addition to the concentrations of progesterone in peripheral circulation, progesterone receptor expression in the endometrium (both luminal epithelium and superficial glandular epithelium) is altered in cows with decreased versus elevated concentrations of progesterone during early postconception. In cows with low postconception concentrations of progesterone, not only are specific nutrient transporters decreased in the endometrium but also progesterone receptor expression is maintained for a longer period of time in the luminal and superficial glandular epithelium. In contrast, elevated concentrations of progesterone, brought about by exogenous treatment, increase nutrient transporter expression and decrease progesterone receptor expression in the luminal and superficial glandular epithelium [24]. Moreover, this downregulation in endometrial progesterone receptors is required to establish uterine and endometrial receptivity to conceptus attachment.

Placental Vascularization

The placenta plays a major role in the regulation of fetal growth. The ruminant placenta is morphologically classified as cotyledonary and histologically as syndesmochorial. On the uterine wall there are specialized areas of endometrium called caruncles, with a button-like appearance [25]. In non-pregnant ruminants, the caruncles are organized in two dorsal and two ventral rows that run lengthwise along the uterine horns. There are approximately 100 caruncles and they can be seen even in uteri from fetal calves [26]. The chorioallantois, which has a flat surface, becomes irregular when it starts to cover the caruncles; this is caused by the growth and expansion of the conceptus on the lumen of the uterus. This process is followed by the recognition of the cotyledons [26]. The caruncular-cotyledonary unit is called a placentome and is formed from the growth and interdigitation of fetal villi and caruncular crypts adopting a convex shape [25]. Contact surface area is enhanced because the cotyledon fingerlike projections enter the crypts formed in the caruncles. Placentomes vary in size, but they are bigger in the horn and decrease in size close to the tip of the horns [26]. The placentome is the primary functional area of physiological exchanges between mother and fetus.

The efficiency of placental nutrient transport is directly related to uteroplacental blood flow [27]. All nutrients and wastes that are exchanged between the maternal and fetal systems are transported via the uteroplacental unit [27, 28]. Establishment of functional fetal and uteroplacental circulations is one of the earliest events during embryonic/placental development [29, 30]. In order to support the exponential increase in fetal growth during the last half of gestation [31], proper growth and development of the uteroplacental vascular bed must occur during the first half of pregnancy [27, 32]. Understanding the factors that impact uteroplacental blood flow will directly impact placental efficiency and thus fetal growth. However, despite intensive research in the area of placental–fetal interactions, the regulators of placental growth and vascularization as well as uteroplacental blood flow are still largely unknown, particularly in cattle.

Limited data on development of the bovine placentomal capillary bed are available. In the cow, cotyledonary growth progressively increases throughout gestation [33, 34]. Histological analysis of capillary bed development in the cow has been performed at mid and late gestation [34]. From mid to late gestation, capillary area density, a measure related to blood flow, decreases by about 30% in caruncular tissue, while it increases about 186% in cotyledonary tissue (Figure 26.1). The numbers of capillaries increase by about 150 and 80% in caruncular and cotyledonary tissue, respectively, during this time. Capillary surface density, a measurement related to nutrient exchange, is also increased in both caruncular and cotyledonary tissues (32 and 172%, respectively), while capillary size decreases 67% in caruncular tissue but increases 71% in cotyledonary tissue from mid to late gestation (Figure 26.1). The pattern of capillary development is very different between the maternal and fetal portions of the placentome possibly due to the energetic demands of these independent tissues that share a similar function – delivery of nutrients to the developing calf.

It is clear that the placenta plays a fundamental role in providing for the metabolic demands of the fetus. Although placental growth slows during the last half of gestation, placental function increases dramatically to support the exponential rate of fetal growth [35, 36]. For example, in sheep and cattle, uterine blood flow increases approximately threefold to fourfold from mid to late gestation [27, 37, 38]. The relationship between uteroplacental blood flow and conceptus size throughout gestation is further defined in the next section.

Maternal Cardiovascular Function

During pregnancy the physiologic state of the dam is associated with significant but reversible alterations to metabolic demand as well as alterations to the endocrine and cardiovascular systems. As previously mentioned, during early gestation (e.g. maternal recognition of pregnancy and conceptus elongation) endocrine secretions of the ovary and conceptus help establish a healthy pregnancy. Moreover, these secretions serve as biochemical cross-talk between

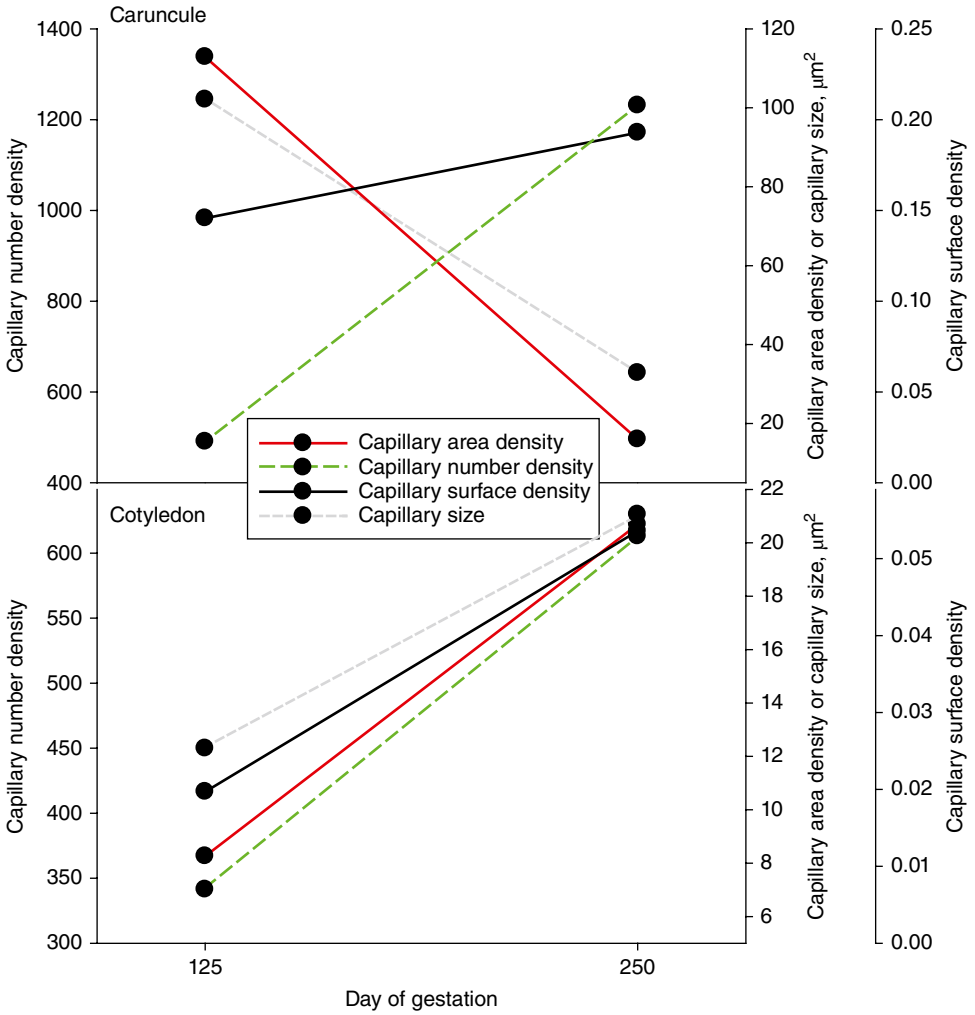


Figure 26.1 Capillary number density, area density, surface density, and size in the maternal (caruncular) and fetal (cotyledonary) portion of the bovine placentome from mid (day 125) to late (day 250) of gestation.

the maternal endometrium and fetal membranes of the placenta during mid to late gestation and help assure proper nutrient and waste exchange during exponential growth of the fetus. Maternal cardiovascular functional capacity changes dramatically during pregnancy, whereby systemic arterial blood pressure and vascular resistance decrease and cardiac output, heart rate, stroke volume, and blood volume increase [39]. Although not all these variables have been determined during bovine pregnancy, several mammalian species including the ewe have shown a decrease in mean arterial pressure in early pregnancy that persists throughout gestation. Moreover, the decrease in arterial pressure (estimated as 5–10%) is minor compared with the approximate 20–30% decrease in total peripheral vascular resistance. Maternal cardiac output has been shown to increase as much as 30–40% in pregnant versus non-pregnant ruminants. Therefore the increase in cardiac output is associated with a dramatic fall in systemic vascular resistance, allowing researchers to characterize pregnancy as a state of systemic vasodilation resulting in profound increases in total systemic flows to all vascular beds. The majority of these studies characterize this relationship by the following equation:

$$\text{Systemic vascular resistance} = \frac{\text{Mean arterial pressure}}{\text{Cardiac output}}$$

Using this equation we can see that the decrease in systemic vascular resistance during pregnancy and the increase in cardiac output (total systemic blood flow) help to maintain arterial pressure. In addition, apart from this relationship, increases in blood volume and activation of the renin–angiotensin system may contribute to maintaining blood pressure during this physiological state of substantial vasodilation; however, limited data exist in cattle. To determine specific contributions to the increase in cardiac output occurring during pregnancy we must first

examine the cardiac output equation, which states that cardiac output = heart rate × stroke volume, where stroke volume equals the volume of blood pumped from one ventricle of the heart with each beat. In the majority of mammalian species, heart rate increases by approximately 15% during pregnancy, which does not fully explain the 30–40% increase in cardiac output. Therefore stroke volume may increase as much as 30–35% during pregnancy and is one of the major contributors to this increase in cardiac output. In sheep treated chronically with estrogen, researchers have shown a similar pregnancy-induced increase in left ventricular heart dimensions and enlargement; therefore the changes to the endocrine system during pregnancy may help mediate the temporal changes to maternal cardiovascular function [39].

The rise in maternal cardiac output during pregnancy is also associated with an increase in plasma and blood volume in cows [40]. This increase in blood volume varies between species and will depend on the nutritional status of the dam as well as singleton versus twin pregnancies. In cattle, blood volume expands by only 10–20% during pregnancy, while in litter-bearing species blood volume may expand by 30–50% during pregnancy. With the increase in plasma volume, the dam must maintain a proper balance of water and electrolyte retention; therefore, similar to the alterations in maternal arterial pressure, this increase in plasma volume will be integrated with the renin–angiotensin system which can serve an additional purpose as an extrinsic modulator of kidney function and urinary secretion. In addition to the dramatic changes in the maternal cardiovascular system during pregnancy, it is even more noteworthy to point out that the majority of mammals will return to non-pregnant levels of cardiovascular function within two to five weeks after parturition. Although lactating, high-producing, non-pregnant dairy cattle will show a substantial increase in cardiac output compared with their non-pregnant, non-lactating counterparts, this redistribution of blood flow during the transition period from the uteroplacental vasculature toward the mammary gland is still a phenomenal physiological feat to allow for peak lactation shortly after parturition.

Maternal Nutrient Partitioning and Endocrinology

Interactions between the maternal endocrine system and the conceptus, and vice versa, are vital to assure continual development of the bovine fetus and healthy offspring fit for life outside the uterus. One example of this interaction is the ability of the bovine conceptus to stimulate mammary growth and development during gestation. This controlled programmed development of the mammary gland allows for synchronization of milk production and secretion with the delivery of a mature calf [41]. Hormonal production by the conceptus may act in a paracrine manner to stimulate its own growth; however, this hormonal production may also interact with the maternal endocrine system to regulate mammary growth, development, and lactogenesis. Secretion of bovine placental lactogen peaks at around 160–200 days of gestation and then remains high until term. Dairy cows have elevated concentrations of bovine placental lactogen compared with beef cows; furthermore, within a subpopulation of dairy cows, a significant positive association has been observed between placental lactogen concentrations during gestation and subsequent milk yield. In addition, production of estrone, conjugated estrone sulfate, and estradiol is observed to increase between days 70 and 110 of gestation. This increase in estrogens should allow for an increased exposure of maternal tissues to estrogenic activity; however, considerable conjugation of estrogen by the uterus and conceptus before transfer into peripheral circulation will decrease maternal tissue exposure. This increase in production should correspond to an increase in aromatase activity as well as estrogen sulfotransferases, generating an estrone sulfate metabolite with low biological activity but a longer peripheral half-life. However, irrespective of the lowered biological activity of conjugated estrogens, the concentration of total estrogen (conjugated and unconjugated) is highly correlated with birthweights and subsequent preweaning growth rates in beef calves, which may be associated with subsequent milk production of the dam. Apart from these associations between conceptus hormone production and subsequent milk production of the dam, it is still important to note that several hormones of pregnancy (progesterone, estrogens, and placental lactogens) regulate cell numbers and vascularity of the mammary gland.

Regulation of nutrient partitioning during pregnancy is an orchestrated event helping to maintain the metabolism of body tissues necessary to support the physiological state [42]. In dairy cows, pregnancy imposes a substantial cost to the animal, so that nutritional plane at the end of gestation is about 75% greater than in a non-pregnant cow of the same weight [42]. After seven months of gestation the fetus will have acquired approximately 40% of its birthweight, while during the last two months of gestation fetal demands for glucose and amino acids are equal to mammary use of nutrients, equivalent to about 5 kg of milk daily [42]. Therefore, in the dairy cow, drying off cows at approximately 60 days before expected calving helps ease the competition between nutrient use for lactation and the major phase of fetal growth.

There is limited information on the specific nutrient requirements of the bovine fetus compared with the extensive amount of research accomplished in the sheep fetus. Gross carbon uptake of the sheep fetus is approximately 8 g/day per kilogram of fetal weight, of which 40% is retained and the remainder is shuttled back to the dam as either carbon dioxide or urea carbon. Apart from glucose, amino acids provide a large portion of the metabolic fuel, estimated at a gross nitrogen accretion rate of 0.65 g nitrogen per kilogram of fetus per day, which is in excess of the average fetal uptake of 1.5 g of nitrogen per kilogram of fetus per day. As mentioned with the carbon excretion via urea, nearly 0.3 g of nitrogen per kilogram of fetus per day is excreted as urea, indicating amino acid catabolism, presumably via gluconeogenic pathways of the fetus and placenta. It is important to note that the influxes of nutrient substrates to meet the needs of fetal oxidative metabolism are also used for fetal anabolism and it has been proposed that specific nutrient substrate fate (catabolism vs anabolism) is controlled autonomously by the fetus. This has led some researchers to propose that the fetus should not be regarded as a "passenger" but rather that it has some self-control or autonomy in its own fate. This is more apparent when we examine some of the changes in the fetus and placenta that allow the fetus to adapt to specific environmental insults bestowed upon the dam.

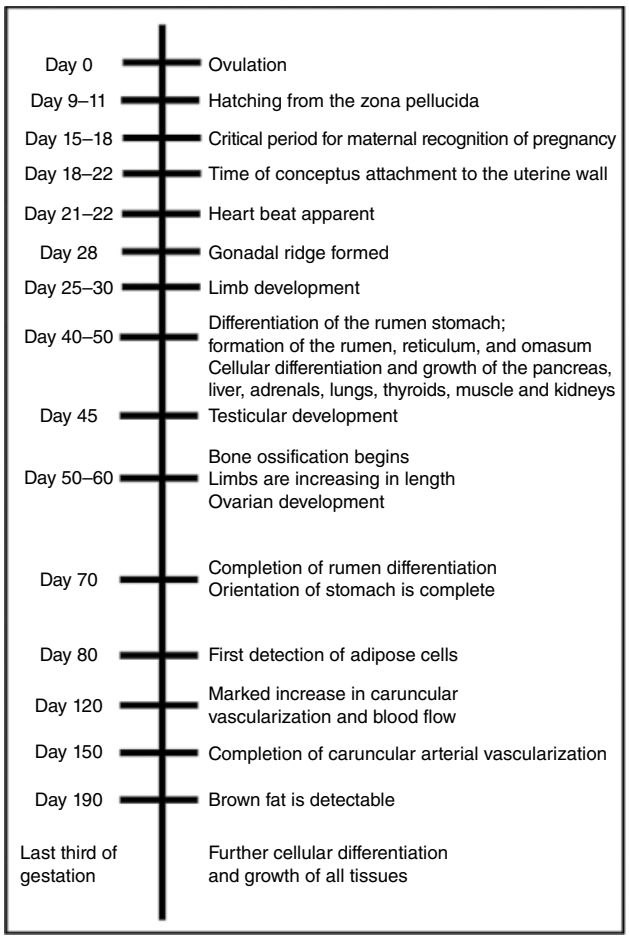


Figure 26.2 Time-line of bovine fetal development. See text for references.

Embryogenesis, Fetal Growth, and Organ Development

The embryonic period in cattle is defined as the time from conception (i.e. single-cell embryo, referred to as a zygote) to the completion of organogenesis. This period typically runs from day 1 to day 42 of gestation and the largest percentage of pregnancy wastage occurs during the embryonic period. The fetal period is defined as the remainder of gestation from day 42 until day 280 when the completion of organ differentiation occurs (Figure 26.2).

Embryogenesis

During early embryogenesis and prior to conceptus attachment, differentiation of the germ cell layers is becoming apparent. The three germ cell layers are referred to as the endoderm, mesoderm, and ectoderm. The endoderm, the innermost layer, will differentiate into the entire alimentary tract, except for part of the mouth and terminal part of the rectum, which are lined by protective involutions of the ectoderm. In addition, the endoderm will develop into the respiratory tract (trachea, bronchi, and lung alveoli), the majority of the endocrine glands, and the urinary systems. The ectoderm is the most exterior germ cell layer and will give rise to the nervous system and the integumentary system. The middle germ cell layer, the mesoderm, will give rise to cardiac, skeletal, and smooth muscle, the circulatory system, as well as the majority of the reproductive tract, excluding the external portions of the genitalia which develop from the ectoderm.

During embryogenesis, an invagination of the oral ectoderm toward the neural epithelium results in a diverticulum referred to as Rathke's pouch. During the formation of this diverticulum, it will lose contact with the oral ectoderm, forming the anterior portion of the pituitary. This anterior lobe is sometimes referred to as the adenohypophysis, because of the glandular derivation of the tissue. Few studies have characterized the formation of Rathke's pouch during early bovine embryogenesis; however, a common disorder known as a residual cleft cyst of the pituitary gland has been recently examined using postmortem magnetic resonance imaging of cattle [43]. Residual cleft cyst of the pituitary was observed in 26–35% of slaughtered

cattle, which showed several morphological similarities to the human disorder known as Rathke's cleft cysts. These structural abnormalities of the pituitary may allow gradual accumulation of endocrine secretions over time and Holstein cattle may be more at risk for developing fluid-filled lesions compared with other breeds. More than likely, these residual pituitary cysts are formed early during embryogenesis and early formation of Rathke's pouch; however, pathological conditions and/or productivity have not been associated with the appearance or relative size of the pituitary cysts in cattle [43].

Sexual Differentiation and the Freemartin

In the realm of bovine fetal organogenesis, no other organ system has garnered as much attention as the development of the reproductive tract. From a sexual differentiation standpoint, the genetic sex of the offspring is established at the time of fertilization when the sperm delivers either an X (female) or Y (male) chromosome to the homogametic oocyte. In cattle, following a few weeks of embryogenesis the process of gonadal sexual differentiation will begin. One of the first steps in sexual differentiation of the gonad involves migration, proliferation, and invasion of primordial germ cells. It is now accepted that the first primordial germ cells also represent hematopoietic stem cells originating from the wall of the allantois as well as the yolk sac of the mesoderm [44]. Hematopoietic stem cells also originate in other parts of the mesoderm and more recent findings suggest that the final proliferative primordial germ cells of the gonad originate exclusively in the aorta–gonad–mesoderm region, while short-lived hematopoietic stem cells of the yolk sac have a restricted differentiation capacity. It is important to note the early common lineage of hematopoietic stem cells (undifferentiated blood cells) and primordial germ cells (undifferentiated gametes). The apparent differences between these cell types in the adult may be due to the presence of surrounding interstitial and stromal cells of the gonad, which help to further differentiate these early hematopoietic stem cells into germ cells. By 23–25 days of embryonic age in cattle, the primordial germ cells are located in the midgut and hindgut in close proximity to the undifferentiated gonad. By 27–31 days of embryonic age these primordial germ cells start to populate the undifferentiated gonad, which rests on the dorsal body wall known as the gonadal ridge. This accumulation of primordial germ cells at the gonadal ridge will continue until day 40 of embryonic age.

Two potential pathways for the transportation of primordial germ cells to the gonadal ridge have been proposed. First, most mammalian species are thought to have active migration of primordial germ cells via a chemoattractant produced by the gonadal ridge. The second pathway involves passive transportation via the bloodstream toward the gonadal ridge, which occurs in avian species and some reptiles. Recent advances in localizing the temporal-spatial distribution of bovine primordial germ cells during early embryogenesis have argued against both of these pathways [45]. The active migration of primordial germ cells may not be necessary because early bovine primordial germ cells (day 18 of embryonic age) have been localized to the proximal yolk sac at a distance of less than 100 μm from the indifferent germinal disk. Therefore focus on the transportation system may be redundant due to the close proximity of the primordial germ cells to the developing gonadal ridge during early development. However, keep in mind that from day 18 to day 25 the growth and elongation of the embryo will continue to move the primordial germ cells further from their point of invasion; therefore some form of transportation and proper signaling from the gonadal ridge of the indifferent gonad would be necessary as embryo length increases.

The embryo develops three separate renal systems: the pronephros, mesonephros, and metanephros. These systems develop in pairs. The pronephros is the most basic of the three excretory organs of the bovine embryo and it will degenerate via apoptosis shortly after formation of the mesonephros (the embryonic kidney). In lower animals, the mesonephros will remain as the adult kidney; however, in the cow the final differentiation of the metanephros will form the functional adult kidney. The mesonephros is also referred to as the Wolffian ducts. At the same time as mesonephric development another pair of ducts will develop next to the mesonephros, referred to as the paramesonephric ducts or Müllerian ducts. At this stage of development the embryo is still uncommitted to sexual differentiation of the reproductive tract [46]. If the genetic sex is male (XY), the paramesonephric ducts will regress and the mesonephric ducts will differentiate to form the epididymis, vas deferens, and seminal vesicles of the male reproductive tract. If the genetic sex is female (XX), the mesonephric ducts will regress and the paramesonephric ducts will differentiate to form the oviducts, uterus, and anterior portion of the vagina.

This process of differentiation starts at the level of the indifferent gonad, where mesothelial cells start a chain reaction of events based on the expression of a transcription factor known as SRY (Sex-determining Region of the short arm of the Y chromosome) [47]. These mesothelial cells or surface epithelial cells of the indifferent gonad infiltrate the gonad during invasion and proliferation of the primordial germ cells and become the gonadal ridge. Furthermore, in the male these mesothelial cells will differentiate into Sertoli cells, and following activation of the SRY gene these Sertoli cells begin to secrete anti-Müllerian hormone. This anti-Müllerian hormone causes the regression of the paramesonephric ducts. The interstitial cells of

the differentiating testis, also known as Leydig cells, begin to secrete testosterone that helps to further differentiate the mesonephric ducts as well as the accessory sex glands of the male. In the absence of the Y chromosome (female genetic sex, XX), the ovaries will begin to differentiate and the lack of anti-Müllerian hormone leads to differentiation of the paramesonephric ducts, while the lack of testosterone leads to the regression of the mesonephric ducts. Several pioneering experiments have further elucidated the mechanisms behind sex differentiation and sex reversal [46]. For example, bilateral castration of the indifferent gonad of either a genetically sexed male (XY) or female (XX) leads to regression of the mesonephric ducts and development of the paramesonephric ducts (phenotypically female irrespective of genetic sex).

The focus on sex determination and sex reversal in the bovine is partly due to the phenomenon known as the freemartin. A freemartin is a sterile heifer born as a co-twin to a bull, and it is explained by the anastomosis that occurs between the placenta of the female and male fetuses. Because of the shared blood supply the sexually differentiating female tract (mesonephric and paramesonephric ducts) is exposed to anti-Müllerian hormone and testosterone from the male co-twin. One of the problems with studying the freemartin is reproducing the syndrome efficiently during an experimental study. For example, the presence of testosterone during critical periods of development will produce a masculinized female fetus (by helping to differentiate a portion of the mesonephric ducts); however, this experimental model is not truly a freemartin because the female fetus produced is not a chimera (containing both XX and XY cell types). This chimerism, presumably from the invasion of male (XY) hematopoietic stem cells or white blood cells from the anastomosis of the placenta, leads to an intersexed gonad, which sometimes contains a mixture of seminiferous tubules of the medulla and follicular pools in the cortex of the gonad. Interestingly, the hematopoietic stem cells may function similar to the primordial germ cells of the developing gonad because of the similarities in the lineage of these cell types. Although the regression and/or rescue of the duct system is primarily under the control of hormonal input, further research is needed to elucidate the formation of the intersexed gonad, which is presumably a function of invasion of XY blood cells of the male twin.

Fetal Growth and Organ Development

The characterization of bovine fetal growth throughout gestation has allowed researchers to determine specific environmental insults that may perturb normal development in utero. In addition to the extrinsic environmental effects altering fetal growth, several inherent intrinsic mechanisms are associated with fetal growth and development. During days 70–100 of gestation, fetal weight across several breeds of cattle has been shown to increase at approximately 10 g/day [48]. Further along in gestation, from days 200 to 250 of pregnancy, the rate of fetal growth increases to approximately 200–300 g/day; however, the absolute growth of the late-term fetus declined to 100 g/day, which may be due to an inherent function of the fetus exceeding the capabilities of uteroplacental exchange near term. Alternatively, the fetus may be secreting or altering its own hormone profiles near term, favoring proper maturation and differentiation of organs over growth.

During these characterization studies, several researchers noted that bovine fetal growth typically lags behind allantoic fluid volume and expansion of the chorioallantoic membrane [48, 49]. As mentioned above, nutrient partitioning by the uteroplacental unit and a continuous supply of nutrients is a prerequisite for fetal growth. The chorioallantoic expansion over the endometrium allows maximal surface area for nutrient exchange during development, which should support the acceleration in fetal growth. A significant positive correlation was observed for total amniotic fluid volume and fetal weight; however, no correlation was observed for total allantoic fluid volume and fetal weight [48]. The cloning of cattle by somatic cell nuclear transfer has allowed researchers to further define this association between fetal fluid homeostasis and fetal and placental development [50]. Cloned bovine fetuses that show abnormal placentation are usually associated with excessive fluid accumulation in the fetal sacs, known as hydrops syndrome. In this experimental model, cloned embryos with hydrops syndrome surviving until mid-gestation show overgrowth of the placentomes, fetal liver, and fetal kidney compared with normal pregnancies [50]. In addition, in several studies utilizing different mammalian species, organomegaly of the offspring's liver, kidney, and heart is typically accompanied by polyhydramnios. Moreover, the underlying mechanisms of fluid sac homeostasis are not fully understood in the pregnant cow and further research is needed to elucidate the proper cause and effect relationship.

Failure of proper organ development occurs in cloned calves that die shortly after birth. Using this experimental model, researchers have examined eight developmentally important genes in six different organs (heart, liver, spleen, lung, kidney, and brain). Of these developmental genes, the kidney was the least affected organ associated with calf mortality, while the heart was the organ most affected by gene dysregulation compared with normal offspring [51]. Of these developmental genes, vascular endothelial growth factor (VEGF) was upregulated in cloned offspring associated with early neonatal mortality. VEGF has been implicated in

stimulating vasculogenesis and angiogenesis to restore oxygen supply to tissues when blood circulation is inadequate. The proper concentrations of VEGF during organogenesis and early organ development are vital for proper establishment of the fetal cardiovascular system. Downregulation of VEGF could lead to a decrease in fetal organ angiogenesis, with fatal consequences for the fetus (pregnancy wastage) or neonate (calf mortality); however, upregulation may result in organomegaly or fetal cardiac dysfunction, with similar fatal consequences to the newborn.

Uterine Blood Flow

Several techniques (electromagnetic probes, heavy water, Doppler) have been used to characterize uterine blood flow in pregnant cows. During early pregnancy and around the time of maternal recognition of pregnancy, uterine blood flow relative to the side of pregnancy (ipsilateral or contralateral) responds differently, which may be due to conceptus secretions during early establishment of pregnancy [52]. Comparing electromagnetic blood flow probes with recent observations using Doppler ultrasonography, we can see a similar increase in uterine artery blood flow during the first trimester of pregnancy (Table 26.1). Moreover, the diameter of uterine arteries remains relatively constant during this period, while the arterial resistance index decreases [53]. During the second trimester of pregnancy, uterine artery blood flow increases from approximately 1–2 l/min to approximately 4–5 l/min (Table 26.2). Moreover, the first signs of increased maternal cardiac output may become apparent during this stage of pregnancy. In addition, uterine blood flow relative to the side of pregnancy (ipsilateral or contralateral) begins to diverge, allowing an increase in oxygen and nutrient delivery as well as waste exchange across the gravid pregnant uterine horn (Table 26.2). In examining Holstein cows over 147 days of gestation, Herzog et al. [54] reported similar total uterine artery blood flow in cows carrying heavy versus light calves; however, at day 175 of gestation, cows carrying heavy calves had an increase in uterine artery blood flow compared with cows carrying light calves. Similarly, Lemley et al. [60] found that maternal nutrient restriction during mid-pregnancy, resulting in growth-restricted fetuses, decreased total uterine artery blood flow compared with adequate-fed pregnant heifers (Table 26.2). This characterization of uterine blood flow versus relative size of the offspring may allow researchers to determine specific time points in gestation where supplements or insults may be used to alter offspring growth and outcome. These pathways will be further discussed in Chapter 27.

The greatest increase in uterine blood flow has been shown in late gestation during the third trimester of pregnancy (Table 26.3). Moreover, this exponential increase in uterine blood flow during the third trimester of pregnancy corresponds to the greatest increase in fetal weight deposition. The variation in reported blood flow during late gestation is a result of laboratory technique as well as individual animal variation. Using electromagnetic blood flow probes, Ferrell and Ford [55] reported uterine blood flow between days 202 and 224 as 4.0 ± 0.5 l/min and between days 230 and 258 as 3.1 ± 0.2 l/min. In a later study using a different technique the same laboratory reported late-gestation ipsilateral uterine artery blood flow at day 226 as 2.9 l/ min and at day 250 as 13.1 l/min. In addition, there was a 4.5-fold increase in ipsilateral blood flow from days 137 to 250 and maternal heart rate was also increased [56, 57]. The next experiment used Charolais cows carrying either Charolais or Brahman fetuses and Brahman cows carrying either Charolais or Brahman fetuses. At day 220 of gestation ipsilateral uterine artery blood flow of Brahman cows carrying Charolais or Brahman fetuses was similar but lower than

Table 26.1 Bovine uterine blood flow (UBF) during the first trimester of pregnancy.

Breed	Stage of pregnancy	Method	Response	Additional response	Reference
Hereford	First 30 days	Electromagnetic blood flow probes	↔ UBF until day 13 Two- to threefold ↑ ipsilateral UBF days 14–18 ↔ contralateral UBF	Days 19–25 ↓ UBF ipsilateral UBF ↑ until day 30 Contralateral UBF ↓	Ford et al. [52]
Hereford	Approximately days 30 and 80	Electromagnetic blood flow probes	Days 31–45, 0.12 ± 0.02 l/min Days 77–92, 0.20 l/min		Ferrell and Ford [55]
Simmental Brown Swiss	Days 30, 60, and 90	Doppler	↔ both arteries Day 30, average 0.10 l/min Day 60, average 0.14 l/min Day 90, average 0.30 l/min	↓ RI by day 60 ↔ diameter	Bollwein et al. [53]

↔, Steady change; ↑, increased; ↓, decreased; RI, resistance index.

Table 26.2 Bovine uterine blood flow (UBF) during the second trimester of pregnancy.

Breed	Stage of pregnancy	Method	Response	Additional response	Reference
Hereford	Approximately days 140 and 180	Electromagnetic blood flow probes	Days 139–155, 2.0 ± 0.6 l/min Days 178–199, 3.2 ± 0.4 l/min		Ferrell and Ford [55]
Hereford	Days 163–166 Days 173–176	Steady-state diffusion; antipyrin solution	Mean 5.9 ± 0.5 ml/min	Mean HR 68.6 ± 2.6 bpm	Ferrell et al. [59]
Simmental Brown Swiss	Days 120, 150, and 180	Doppler	Ipsilateral day 120, 0.7 l/min Contralateral day 120, 0.4 l/min Ipsilateral day 150, 1.7 l/min Contralateral day 150, 0.9 l/min Ipsilateral day 180, 4.0 l/min Contralateral day 180, 1.3 l/min		Bollwein et al. [53]
Holsteins	Days 147 and 175	Doppler	↑ Total UBF in heavy vs light cows: Light cow: day 147, 2.6 l/min Heavy cow: day 147, 3.4 l/min Light cow: day 175, 4.2 l/min Heavy cow: day 175, 5.4 l/min	At day 147, cows had similar total UBF when carrying heavy or light calves, but at day 175, cows carrying heavy calves had ↑ blood flow vs light calves	Herzog et al. [54]
Angus Brahman heifers	Day 175	Doppler (total UBF; summation of left and right uterine arteries)	Angus, 5.6 l/min Brahman, 3.2 l/min Control, 5.7 l/min Nutrient restricted, 3.0 l/min	Nutrient-restricted heifers had ↓ blood flow compared to control-fed heifers	Lemley et al. [60]

↑, Increased; ↓, decreased; HR, heart rate.

in Charolais cows carrying Charolais or Brahman fetuses. However, with regard to the Charolais cows, those carrying Charolais fetuses had increased blood flow compared with those carrying Brahman fetuses. This study shows that late-gestation uterine blood flow and function of the uteroplacental unit may be the limiting factors for fetal growth [38]. The third study used two different breeds (Charolais and Hereford) but also looked at single versus twin fetuses. At day 190 of gestation, there was a decrease in ipsilateral uterine artery blood flow for Hereford versus Charolais cows. Another observation from this experiment was that the cows carrying twins had decreased blood flow compared with cows carrying singletons regardless of cow breed. Therefore twin pregnancies with reduced birthweight compared with singletons may be due to the reduced blood flow per fetus that was observed [58].

More recent studies have been conducted utilizing Doppler ultrasonography to examine uterine blood flow during late gestation. Bollwein et al. [53] reported ipsilateral and contralateral uterine artery blood flow on days 210, 240, 270, and 284 of gestation. They observed an increase in blood flow and arterial diameter during gestation and decreased resistance of the uterine artery during the first eight months of gestation while the last

Table 26.3 Bovine uterine blood flow (UBF) during the third trimester of pregnancy.

Breed	Stage of pregnancy	Method	Response	Additional response	Reference
Hereford	Approximately days 210 and 240	Electromagnetic blood flow probes	Days 202–224, 4.0 ± 0.5 l/min Days 230–258, 3.1 ± 0.2 l/min		Ferrell and Ford [55]
Brahman Charolais	Day 220 ± 0.4	D_2O (ipsilateral) infusion	↑ Ipsilateral UBF in Charolais vs Brahman	Charolais carrying Charolais fetuses ↑ blood flow vs Charolais carrying Brahman fetuses	Ferrell [38]
Charolais Hereford	Day 190 ± 0.5	D_2O (ipsilateral) infusion	↓ UBF Hereford 4.8 vs Charolais 7.1 l/min	↓ UBF in cows with twins 5.2 vs single 6.7 l/min	Ferrell and Reynolds [58]
Simmental Brown Swiss	Days 210, 240, 270, and 282	Doppler	Ipsilateral day 210, 5.0 l/min Contralateral day 210, 1.3 l/min Ipsilateral day 240, 6.0 l/min Contralateral day 240, 2.8 l/min Ipsilateral day 270, 10.5 l/min Contralateral day 270, 3.9 l/min Ipsilateral day 282, 13.1 l/min Contralateral day 282, 4.5 l/min	↑ UBF and diameter during gestation ↓ RI during first eight months and ↔ after	Bollwein et al. [53]
Holstein	Days 203, 231, 259, and 273	Doppler	Light cow: day 203, 6.1 l/min Heavy cow: day 203, 8.7 l/min Light cow: day 231, 10.5 l/min Heavy cow: day 231, 13.1 l/min Light cow: day 259, 13.6 l/min Heavy cow: day 259, 16.9 l/min Light cow: day 273, 14.1 l/min Heavy cow: day 273, 19.2 l/min	Linear ↑ in total UBF 3.0 to 16.9 l/min ↑ Total UBF in heavy cows vs light cows. Similar-weight cows with heavy calves had ↑ total UBF vs light calves	Herzog et al. [54]
Holstein heifers	Days 180, 210, 240, and 262	Doppler (total UBF; summation of left and right uterine arteries)	Control heifer: day 210, 4.6 l/min Melatonin heifer: day 210, 6.7 l/min Control heifer: day 240, 7.2 l/min Melatonin heifer: day 240, 8.8 l/min Control heifer: day 262, 7.8 l/min Melatonin heifer: day 262, 10.1 l/min	Linear ↑ in total UBF from 3.1 to 9.0 l/min from day 180 to 262 of pregnancy Melatonin-treated heifers had ↑ total UBF vs control heifers	Brockus et al. [61]
Beef heifers and cows	Days 210–240	Doppler (total UBF; summation of left and right uterine arteries)	Control heifer: 5.3 l/min Melatonin heifer: 6.4 l/min Control cow: 7.0 l/min Melatonin cow: 9.0 l/min	Beef cows had ↑ UBF vs beef heifers Melatonin cattle had ↑ UBF vs control cattle	McCarty et al. [62]

↔, Steady change; ↑, increased; ↓, decreased; RI, resistance index; D_2O, deuterium oxide.

month arterial resistance was similar. The second experiment by Herzog et al. [54] measured blood flow on days 203, 231, 259, and 273 of gestation. Throughout this experiment they observed a linear increase in total uterine artery blood flow. Also, heavy cows had a greater increase in blood flow compared with light cows; however, similar-weight cows carrying heavy fetuses had greater blood flow than those carrying light fetuses. In addition to characterization studies, Brockus et al. [61] and McCarty et al. [62] observed increased uterine artery blood flow in Holstein heifers and beef cattle supplemented with melatonin during the last third of pregnancy (Table 26.3). These changes in uterine blood flow responses during pregnancy have direct consequences on the growth and development of offspring, as will be discussed in Chapter 27.

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27

Fetal Programming

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Introduction

Irrespective of an animal's genotype, an environmental stimulus or insult during a critical period of development can program the observed phenotype of an animal. Importantly, this exposure may establish a permanent phenotype throughout the remainder of an animal's life, which can have adverse consequences for milk production, carcass yield, feed efficiency, and/or reproductive function. This process of permanently altering an animal's phenotype through environmental stimuli has been referred to as the developmental programming hypothesis. For example, two animals possessing the same genotype but raised in different environments are expected to have differing lifelong phenotypic characteristics, which is further explained by developmental plasticity. Importantly, the magnitude of phenotypic change is vastly different between these two animals possessing the same genotype if their exposure to different environments occurred while they were embryos, fetuses, calves, weaned heifers, or mature cows. Furthermore, the changes to these animals' developmental trajectory and any lasting consequences are thought to be greatest in embryos and fetuses, with decreasing developmental plasticity with increasing animal age. The study of developmental programming during the fetal period, an age of high developmental plasticity (Figure 27.1), is known as fetal programming.

Studies from different laboratories have shown overwhelming support for the concept of fetal programming by reporting a strong association between birth weight and lifelong developmental consequences. For example, low birth weight offspring are at an increased risk of morbidity and mortality, slowed postnatal growth, poor body composition (increased fat and reduced muscle growth), metabolic disorders, cardiovascular pathologies, and dysfunction of several organs, such as the ovaries, testes, mammary gland, and gastrointestinal tract [1, 2]. Livestock are specifically at risk due to poor nutritional environments during pregnancy, such as breeding of young, growing, peripubertal dams that are competing for nutrients with the fetus. In addition, poor pasture conditions or environmental heat stress in relation to seasonal breeding can specifically decrease nutrient availability for both dam and fetus during critical periods of development. Although these initial fetal programming studies focused exclusively on offspring (fetal or birth) weight, we now understand that multiple measurements of offspring size at birth can predict their developmental trajectory. Therefore phenotypic changes in cattle production as a result of fetal programming may be independent of calf weight at birth. This is evident when environmental insults occur during early pregnancy alone, such that birth weights are unaltered but evident phenotypic changes still impact calf production.

Insufficiencies during pregnancy, resulting in reduced fetal growth and development, are detrimental to all livestock species, where the newborns represent the next generation of meat and milk-producing animals. Several animal models of fetal and placental growth restriction (e.g. maternal nutritional plane, maternal age, heat stress, hypoxic stress, and fetal number) have been developed to better unravel the relationship between uterine blood flow and offspring development [3–6]. Establishment of functional fetal and placental circulation is one of the earliest events during conceptus development [7, 8] and the exponential increase in placental exchange is vital for maintaining the exponential growth and development of the fetus during the last half of gestation [9]. Therefore understanding the impacts of maternal environment on placental function is especially relevant, as the majority of mammalian livestock raised for red meat production spend 30–40% of their life being nourished by the placenta. The percentage of total time in each phase of beef

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

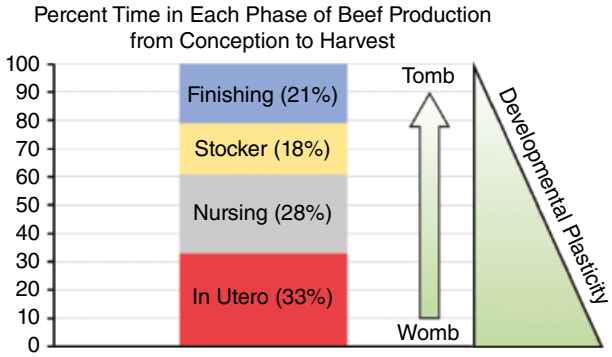


Figure 27.1 Percentage of total lifetime in each phase of beef production from conception to harvest (womb to tomb). Nearly a third of life is spent developing in utero, when the fetus is most vulnerable to the environment because of increased developmental plasticity.

production from conception to harvest is depicted in Figure 27.1. In addition, the amount of developmental plasticity of offspring varies during their lifespan, with maximal influences of developmental programming occurring during the embryonic and fetal stages of life (Figure 27.1).

Adaptations During Pregnancy: Fetal and Placental Development

The embryonic period in cattle is defined as the time from conception to the completion of organogenesis. This embryonic period typically runs from day 1 to 42 of gestation, with the largest percentage of pregnancy wastage occurring during this period of development. The fetal period is defined as the remainder of gestation from day 42 until day 280 when the completion of organ differentiation occurs (see Figure 26.2). The characterization of bovine fetal growth throughout gestation has allowed researchers to hypothesize phenotypic changes to offspring that experience specific periods of environmental insults that may perturb normal development in utero. For example, nutrient deprivation or heat stress during days 60–120 of pregnancy will undoubtedly have different impacts on fetal development compared to the same environmental insults from day 180 to 240 of pregnancy. This is where timing becomes a critical component of fetal programming outcomes. Offspring growth in utero is sensitive to direct and indirect effects of the maternal environment, particularly during the early stages of embryonic life [10]. During early pregnancy hormone secretions from the ovary and conceptus help establish a healthy pregnancy. During mid to late pregnancy the dam undergoes significant but reversible changes in physiology to help maintain the increase in metabolic demand. Of these maternal changes, adequate blood flow to the reproductive tract is critical for normal development of the offspring. In addition, the magnitude decrease in blood flow is reflective of the magnitude decrease in fetal weight. In all domestic livestock species, offspring born at average weight have an increased chance of survival compared with those born below average weight. While the literature is booming with increasing evidence of how nutrient restriction or overfeeding impairs several physiological parameters, fewer concentrate on enhancing postnatal growth in livestock species.

Several animal models of fetal programming have been extensively studied in the ewe [6], and extrapolation to cattle should be minimal because of the drastic differences in placental development between sheep and cattle. In the ewe, the placenta reaches its maximum size during the first two-thirds of gestation, while approximately 90% of fetal growth occurs during the last third of gestation [9]. In contrast, the bovine placenta continues to increase in size exponentially as gestation proceeds; however, the exponential rate in growth of the bovine fetus is much greater compared to the growth of the placenta [11]. The placenta is involved in transporting nutrients and wastes between maternal and fetal circulation and altered placental function has been associated with abnormalities in fetal development. Fowden et al. [12] reviewed key factors affecting placental nutrient transfer capacity, which were size, nutrient transporter abundance, nutrient synthesis and metabolism, and hormone synthesis and metabolism. Large increases in blood flow to the reproductive tract are necessary to support both nutrient and waste exchange between the mother and offspring. This relationship is best depicted by placental efficiency calculations (Figure 27.2).

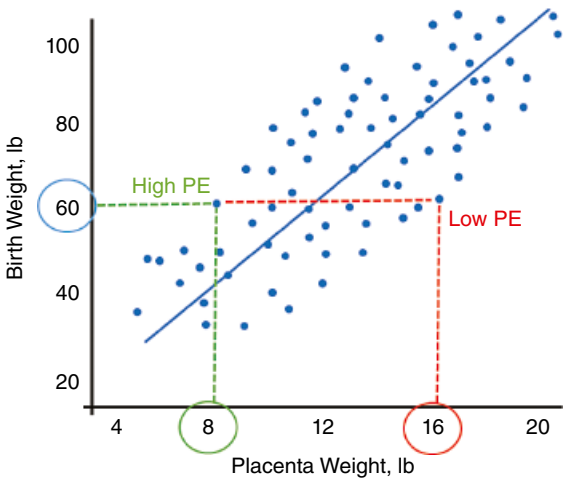


Figure 27.2 Positive association between calf birth weight (y-axis) versus placenta weight (x-axis). Placental efficiency is calculated as birth weight divided by placental weight (pounds of calf that can be grown per pound of placenta). The green lines depict a high placental efficiency, while the red lines depict a low placental efficiency, even though both calves weighed 60 lbs at birth.

Placental efficiency is calf birth weight divided by placental weight and explains the pounds of calf that can be grown per pound of placenta. Undoubtedly, a strong positive relationship exists between calf birth weight and placenta weight; however, focusing on the calves born above or below this trend line (Figure 27.2) shows that some calves are born from an efficient placenta and other from an inefficient placenta. One of the most important indicators of placental efficiency is uterine and umbilical blood flow. Using Doppler ultrasonography researchers have investigated alterations in blood flow to the reproductive tract during specific windows of development. Several environmental factors have been shown to negatively impact placenta development and blood flow during pregnancy, all of which can hinder offspring health and vigor.

Maternal Nutrient Restriction

Poor forage quality in grazing systems can negatively impact the nutritional intake of beef cattle. Pregnant beef cows grazing poor forage can alter fetal growth during increased periods of developmental plasticity. Thus, provisions from the environment can program these offspring to experience changes in mortality and morbidity rates, slowed postnatal growth, altered carcass weights, and meat quality characteristics [13]. The relationship between maternal nutritional plane during late gestation and calf mortality was examined as early as 1975, where maternal nutrient restriction for 100 days prepartum decreased calf birth weight by 7% and increased calf mortality rate by 10%, while an additional 20% of calves died between birth and weaning due to scours [14]. The direct effects of nutritional plane on offspring production characteristics are dependent on the timing of insult and magnitude of nutrition deprivation in relation to fetal and placental development [15]. In dealing with timing, it is also important to consider the separation of prenatal versus postnatal maternal factors that may influence these developmental programming responses. For example, changes in meat quality of offspring born to dams experiencing a late pregnancy maternal nutrient restriction may carry over into the early lactation period of this dam, preventing researchers from identifying the important time of maternal nutritional insult that led to the negative outcomes of the offspring. This is vital when considering the most economically feasible therapeutic interventions to mitigate negative developmental programming outcomes.

The adaptations of the placenta during maternal nutrient restriction are incomplete and less is known about specific differences among breeds of cattle. Normal physiology such as gestation length, fetal growth, placentome weight, and even uterine blood flow can differ substantially between breeds of cattle [16, 17]. Therefore the magnitude of fetal programming is expected to be breed dependent and further research is needed to identify when and with what breeds interventions should be sought. Researchers have examined the effect of early to mid-gestational nutrient restriction on uterine blood flow and fetal development in Brahman and Angus heifers [18]. In this study, heifers were restricted to 60% of net energy requirements for gestating cattle from days 50 to 180 of pregnancy. This early to mid-pregnancy nutrient restriction decreased uterine artery blood flow and fetal weight at day 180 of pregnancy irrespective of heifer breed. Moreover, the efficiency of uterine artery blood flow relative to fetal weight was improved in nutrient-restricted dams versus adequate-fed dams. A similar response was observed in Brahman heifers irrespective of nutrient restriction, signifying a lesser amount of uterine blood flow needed to grow the same weight fetus from a Brahman dam as compared to an Angus. A portion of these responses has been associated with increased placental efficiency in nutrient-restricted dams and Brahman dams [18].

Apart from nutritional management during pregnancy, researchers have examined heifer development practices and season on uterine artery blood flow during mid to late gestation. For example, beef producers opting for low-input forage-based replacement heifer management programs see lighter weights at breeding, with some heifers reaching only 50–55% of expected mature body weight at breeding versus a traditionally recommended target weight of 60–65% of expected mature body weight. Heifers developed on low-input management schemes until confirmation of pregnancy (days 30–45 of gestation) showed no compromise in uterine blood flow or calf birth weights compared to conventionally developed heifers. Moreover, the volume of late gestation uterine artery blood flow relative to maternal body weight was significantly increased in low-input heifers versus conventionally developed, which may be a compensatory mechanism to safeguard fetal growth and development [19]. In addition to low-input heifer development programs, calving season is expected to alter uterine artery blood flow, as differences in postpartum anestrus interval, conception rates, and weaning weights have been reported between fall and spring calving herds [20, 21]. A portion of these responses could be programmed in utero via changes in nutrient and waste exchange between dam and fetus.

Offspring Performance

Quantitatively, meat production is dependent on the efficient development of skeletal muscle. The amount of meat produced is dependent on the amount of muscle tissue an

animal deposits prior to slaughter. Genetic selection has resulted in livestock that are leaner and more efficient at muscle deposition than ever before. Still, the biology of tissue prioritization leaves skeletal muscle development susceptible to environmental and developmental cues in order to ensure that the needs of more vital systems and organs are satisfied before muscle receives its share of substrates to support growth. As meat production becomes more refined and global markets become more competitive, US producers will rely on innovative technologies and novel management strategies to maximize the efficiency, quantity, and compositional quality of muscle growth in the pre- and postnatal phases of production. The amount and distribution of muscle and adipose tissue (fat) on a beef carcass are the primary determinants of the United States Department of Agriculture (USDA) yield and quality grades. These metrics provide economic incentives for beef cattle producers and effectively estimate the amount and quality of boneless closely trimmed retail cuts that the carcass will ultimately produce. This gridded valuation process occurs at the end of the meat production cycle; however, the tissues that contribute to the final carcass have embryonic and fetal origins that are extremely impressionable during the period of prenatal growth and development.

The events and environmental milieu encountered by the developing offspring during these critical prenatal developmental windows establish the metabolic disposition of muscle and adipose tissue for the postnatal stages of production. While there are obvious overt signs of nutrient restriction during gestation such as low birth weight or failure to thrive, there are also underlying tissue-level molecular paradigms that persist despite the best postnatal management and nutritional practices. This is due to the establishment of metabolic predispositions that are less responsive to postnatal nutritional regimens. Therefore the potential to manipulate the representative cells that comprise the muscle, adipose, and connective tissues that make up the muscles and ultimately the meat products that arise from them is highest during the prenatal phase and diminishes over time.

Muscle growth and development begins in utero and this period represents, in earnest, the beginning of the beef production cycle. The relative preponderance, distribution, and composition of skeletal muscle are established prior to birth and then interact with the postnatal environment to determine final meat composition, yield, and quality. While skeletal muscle, as the precursor for meat, is of the utmost economic importance for producers and packers, it has a lower physiological priority compared to other more vital organs and tissues in the developing animal. This makes it particularly vulnerable to perturbations in the metabolic environment in utero. Total muscle fiber number is established by day 90, 105, and 180 of gestation in pigs [22], sheep [23], and cattle [24], respectively. This means that beef cattle, like all mammals, are born with the total number of muscle fibers that they will have at their disposal for postnatal growth and development, and all subsequent growth will be due to longitudinal and radial hypertrophy of these existing muscle fibers. Because muscle growth potential is positively correlated to muscle fiber number [25], and because hyperplasia does not occur postnatally [26, 27], the prenatal window must be deliberately managed to optimize the potential for high yield and high-quality carcasses. Moreover, precision management approaches to maximize postnatal muscle growth must consider the in utero developmental window.

Adipose tissue is intricately involved in whole-body energy balance and the prenatal period establishes a preparatory metabolic program so that the offspring can effectively manage the nutrients that it anticipates will be available in postnatal life. This includes several layers of transcriptional regulation that set up a metabolic program that loses plasticity in anticipation of a restricted postnatal environment similar to the prevailing in utero environment [28]. This can lead to a metabolic mismatch and undesirable carcass development in postnatal life when nutrients are not provided in congruence with the established restricted metabolic program. These offspring become particularly frugal with nutrient utilization and may experience a period of transient compensatory growth that places them on par with their non-restricted contemporaries in terms of weight. However, in many cases the composition of the catch-up growth is adipose tissue, and as a result their metabolic predisposition is toward nutrient storage as lipids in adipose tissue instead of using the energy and metabolic substrates for lean muscle growth and development. It is important for beef producers and packers to understand the impacts of amount of growth versus composition of growth on the final yield and quality of their retail product.

In the Brahman vs Angus nutrient restriction study described above, a subset of the Angus and Brahman heifers were allowed to calve, and postnatal growth was followed through to weaning (unpublished observations). Most postnatal measurements of growth were unaffected by maternal nutrient restriction from days 50 to 180 of pregnancy. However, heart girth was increased in calves born to nutrient-restricted versus adequate-fed dams, which may show in utero overcompensation of fetal growth when nutrient-restricted dams are realimented to adequate nutrition during late pregnancy. These cattle were not carried out to harvest, however, and that would be the defining determination of how fetal nutrient restriction impacted the quantity and quality of muscle and adipose tissue growth in the postnatal phase.

Maternal Melatonin Supplementation

The amplitude of melatonin secretion has been associated with improved oxidative status and altered hormone metabolism in rats and sheep, as well as altered cardiovascular function in several mammalian species [29–31]. Some studies have shown that melatonin partially regulates blood pressure and blood flow [32]. Melatonin has both direct and indirect effects on the cardiovascular system and may cause either arterial vasodilation or vasoconstriction depending on the origin of the blood vessel under investigation. Taking into account the above physiological responses, which can be partially altered by peripheral concentrations of melatonin, researchers have examined the effects of melatonin supplementation on uteroplacental development and functional capacity. Specifically, uterine artery blood flow was increased in Holstein heifers supplemented with 20 mg of dietary melatonin from days 190 to 262 of gestation [33]. Surprisingly, calf birth weights were not different between treatments; however, calf body weight at nine weeks of age was increased in calves born to melatonin-supplemented dams vs control dams [34]. Therefore, similar to other pregnancy models, an increase in uteroplacental blood flow during mid to late pregnancy is associated with alterations in postnatal offspring growth and development. This is apparent in the dairy heifer study as calves were removed from dams, managed identically, and fed a similar milk replacer and starter diet prior to weaning at eight weeks of age. Therefore postnatal maternal factors were removed from this dairy project, allowing researchers to propose direct fetal programming responses.

Because of the observed differences in dairy calf body weights, a similar melatonin supplementation experiment was replicated in beef cows. In this follow-up study, heifers and cows were assigned to one of two treatments: melatonin implants (n = 29) or no melatonin implant control (n = 28) starting on day 180 of gestation and ending on day 270 [35]. As expected, uterine artery blood flow was increased in commercial beef heifers and cows supplemented with melatonin during the last third of pregnancy. Similar to the dairy heifer study, beef calf birth weights were not different; however, a 57-lb increase in weaning weights was observed in calves born to melatonin-supplemented dams versus control [35]. Although similar results to the dairy study, it is important to note that postnatal maternal factors (e.g. postpartum cow health, colostrum composition, and lactational performance) could be contributing to the increased weaning weight of calves born to melatonin-supplemented dams. Taken together, these results in both cattle studies following melatonin supplementation have allowed researchers to speculate on potential circadian alterations of the reproductive tract during pregnancy. For example, the rhythms generated from circulating concentrations of melatonin could mediate circadian rhythms in the placenta and developing fetus during pregnancy.

The modulation of reproductive functionality and physiology by melatonin has a considerable impact on the postnatal growth of muscle and adipose tissue. Prenatal influences may include direct and indirect mechanisms. In skeletal muscle, binding of melatonin to the melatonin receptor stimulates glucose transport into muscle cells [36], increases the number of satellite cells, reduces inflammation, and inhibits apoptosis by modulating associated signaling pathways, allowing for cell regeneration [37]. To determine the underlying etiology of the differences in weaning weights reported above, loin muscle tissues were dissected from a subset of fetal calves collected via cesarean section. Fetal loin muscle weight and length tended to be increased in calves from melatonin-treated dams compared to their control counterparts. In addition, fetuses from melatonin-treated dams had increased expression of adenosine monophosphate-activated protein kinase-α (AMPK), which is involved in energy and protein metabolism. At the histological level, the ratio of Pax7-activated myonuclei to total myonuclei (PAX7:Nuclei) tended to increase in fetuses collected from melatonin-treated compared to control dams, which is indicative of satellite cell activation, a necessity for pre- and postnatal muscle growth. Moreover, these fetuses exhibited decreased expression of adiponectin (ADIPOQ), CCAAT enhancer binding protein alpha (CEBPA), proliferator activated receptor gamma (PPARg), and stearoyl-CoA desaturase (SCD) in perirenal adipose tissue, which is indicative of reduced adipogenesis and lipid metabolism in these offspring. We hypothesized that the improved metabolic status of loin muscle coupled with the decrease in adipogenic gene expression resulted in calves from melatonin-treated dams having improved potential for lean muscle accretion and reduced overall adiposity during postnatal development.

To test this hypothesis, the remaining subset of treated dams were allowed to carry their offspring to term and, while there were no differences in birth weight, loin muscle biopsies were collected from the offspring at birth and throughout the nursing phase to garner a longitudinal perspective of molecular muscle growth over time. Calves from melatonin-treated dams have a tendency for increased AMPK relative expression at birth and exhibited increased myogenic determination factor 1 (MyoD), insulin-like growth factor-1 (IGF1), and IGF2 expression in biopsies collected at day 195 of age. This molecular signature indicates an enhanced myogenic paradigm in the offspring from melatonin-treated dams that persisted at least up until weaning and may explain

the increased weaning weight observed in this treatment group compared to the control group.

Epigenetics and Fetal Programming

The term epigenetics has classically been defined as heritable changes in gene function without a change in the DNA sequence itself. The prefix "epi-" is derived from a Greek word, meaning "on," "above," or "over." Therefore epigenetic modifications can literally be interpreted as modifications occurring on top of genes. Among the most studied types of epigenetic modifications are DNA methylation and histone modifications. DNA methylation is defined as the addition of a methyl group to the five position of a cytosine. Histone modifications are post-translational modifications of histone proteins, some examples of which include acetylation, methylation, and phosphorylation. Epigenetic modifications fundamentally exist to program cell-specific functions. Though every cell in the body possesses the same underling genetic sequence, cells throughout the body have vastly differing functions. Therefore the cell must package genetic material in a way that transcription factors can access necessary portions of the genome and condense or "pack away" unnecessary portions. Open and active portions of the genome are referred to as euchromatin, while condensed and inactive portions are referred to as heterochromatin. Not only do epigenetic modifications function to "package" and "unpackage" portions of the genome for cell-specific function, but also they act to alter gene activity to adapt cells and collectively the body to a changing environment. Specifically, epigenetic modifications can result in increased or decreased transcription of genes, thereby increasing or decreasing the products of those genes. For example, increased DNA methylation within the promoter region of a gene typically results in a conformational change that prevents transcription factors from interacting with the gene, resulting in gene silencing. The epigenome is most sensitive to change during prenatal and early life development. This likely plays a role in decreasing developmental plasticity as an animal ages, as noted in Figure 27.1. Due to the sensitivity of the epigenome during prenatal development, alterations in fetal environment can result in programming of the epigenome and in turn the transcriptome. This relationship is the foundation of proposed epigenetic mechanisms controlling prenatal programming. Furthermore, prenatal programming and corresponding epigenetic modifications are believed to influence subsequent generations. Specifically, prenatal programming influences not only the developing fetus, but also the developing gametes belonging to that fetus (Figure 27.3). Therefore the experience of one individual in utero has the potential to impact the phenotypes of subsequent generations, though it is largely unclear how epigenetic patterns are maintained through the process of epigenetic reprogramming following fertilization.

Epigenetic modifications have been primarily studied in humans and rodents. However, recent literature has emerged evaluating the role of epigenetic modifications in controlling bovine phenotypes. Epigenetic modifications in cattle have been associated with differences in pathology [38], muscle biology [39], temperament [40], milk yield [41], fertility [42], and tropically adapted breed types [43]. However, limited research has been conducted specifically evaluating the role of epigenetic modifications in prenatal programming of postnatal phenotype of cattle. Genome-wide differential methylation of genes related to immune function, behavior, stress response metabolism, and cell signaling was identified in leukocytes of calves that

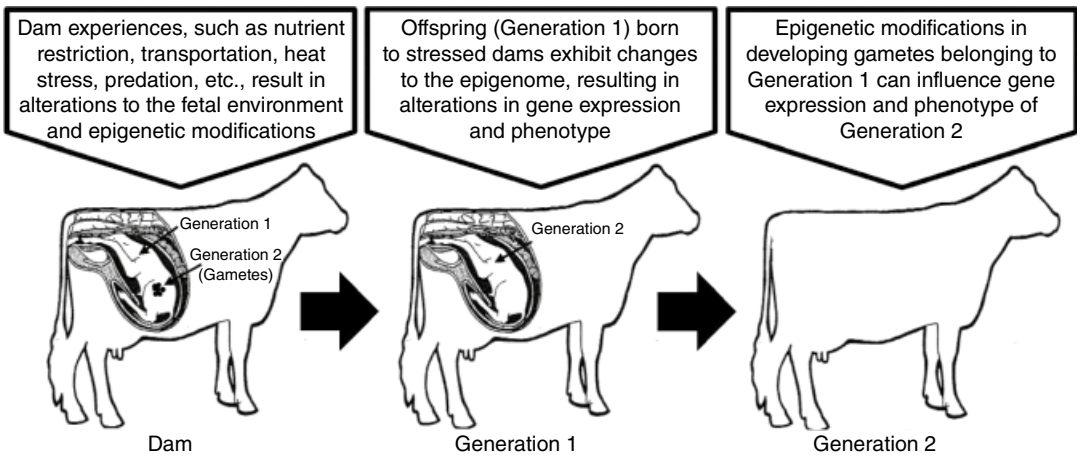


Figure 27.3 Model of prenatal programming in cattle.

experienced prenatal transportation stress [44]. Additionally, the *MIMT1* gene was found to be differentially methylated in placental tissue of intrauterine growth-restricted bovine fetuses [45]. Our current understanding of epigenomic and transcriptomic regulation of phenotype indicates a mechanistic role of epigenetics in prenatal programming. Though great attention has been previously placed on identifying gene markers associated with phenotypic characteristics and conditions in cattle, current literature indicates that more attention should be placed on epigenetic modifications which regulate the expression of such genes. By better understanding mechanisms controlling prenatal programming, we can better understand how to improve bovine production and management systems.

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Breeding and Health Management

28

Biosecurity for Beef and Dairy Herds

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Introduction

The goal of dairy and beef breeding operations is to produce offspring for future production in the forms of meat, milk, or genetics. Subsequently, reproductive diseases can have detrimental effects on the reproductive efficiency of dairy and beef breeding herds. There are many infectious diseases that affect reproductive performance in the bovine, including viral, bacterial, and parasitic. Specific pathogens may cause a range of clinical manifestations, from metritis and infertility to abortion and perinatal loss.

Biosecurity measures for a livestock operation are those taken to prevent the introduction or reintroduction of infectious disease agents into susceptible populations. While many biosecurity recommendations are good general management practices that can be applied across most operations, other recommendations should be more specifically tailored to an individual premises or group of animals.

Biosecurity measures are undertaken at national, state, and local levels. At the national level, biosecurity plans are often mandated by regulatory agencies such as the United States Department of Agriculture (USDA) and the United States Department of Homeland Security (USDHS) to protect livestock resources and consumer interests. Such plans are aimed at preventing the introduction of foreign animal diseases and other diseases of high economic consequence. The entry of animals, animal byproducts, germplasm (semen, embryos), and feed and feed products is carefully controlled at the national border. Producers and veterinarians participate at this level by adhering to recommendations with regard to international travel and by reporting unusual or suspicious conditions to their state or federal animal health official.

Biosecurity plans at the state level may focus on diseases targeted for eradication and control across state lines, such as brucellosis. State plans can also target diseases of regional significance, such as trichomoniasis, and are regulated through the state animal health official, most commonly the state veterinarian. Individual states may have different regulations for interstate animal imports, and both producers and veterinarians are responsible for knowing and adhering to a state's specific animal health regulations. The issuance of certificates of veterinary inspection (CVIs) is one example of an important tool used to prevent the spread of livestock diseases across state lines. Within states, an increasing number of states are also requiring intrastate or transfer of ownership testing of cattle for diseases such as trichomoniasis, and mandatory reporting of infectious disease diagnoses such as bovine viral diarrhea and Johne's disease.

While disease control measures at the national and state levels are critical to the protection of the overall US livestock population, individual producers and veterinarians have the greatest influence over the implementation of local biosecurity measures. It is ultimately individual livestock producers who are responsible for protecting their own herd against infectious reproductive diseases. Local biosecurity plans focus on preventing or controlling the spread of disease between operations and take into account endemic diseases and conditions unique to that population of animals. Local plans should consist of biosecurity measures as well as biocontainment measures – those taken to prevent or decrease the spread of a disease among groups of animals within an operation. Biological risk management (BRM) is the term used to describe the overall awareness and management of the risk of a pathogen or disease entering and spreading throughout a population. BRM often involves the evaluation, management, and reduction of pathogens that are already present in an operation.

Some challenges to recognizing reproductive inefficiencies in a breeding herd include the lack of individual animal identification and good reproductive records, the imperfection of

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

diagnostic tests, and failure to identify the etiologies of abortions and perinatal losses. Furthermore, the intensive management of cattle in the USA, whether it is beef on pasture or dairy in confinement, creates challenges to ideal disease control conditions. Such challenges reinforce the importance for cattle producers to have a herd-specific biosecurity plan in place to protect their herd from reproductive diseases.

Developing a Biosecurity and Biocontainment Plan

A properly performed biosecurity risk analysis will help determine which biosecurity and biocontainment measures are appropriate for a specific operation. While general biosecurity measures are directed at non-specific pathogen threats, a biosecurity risk analysis can be agent specific, industry specific, or event specific [1]. Examples would be a Johne's disease herd risk assessment and management plan, a feedlot biosecurity analysis, and a biosecurity risk assessment for livestock exhibitions, respectively. Risk analyses can also be qualitative or quantitative, depending on the herd information available and desired outcome of the assessment. Many epidemiological tools are available to estimate the risk of specific disease transmission in a population, including statistical simulations and mathematical modeling [2, 3]. Such risk assessments should be made using multiple sources of information, such as targeted producer surveys and diagnostic testing, to predict probabilities of disease transmission, prioritize resources, or monitor progress following changes in management [4].

A biosecurity risk analysis approach involves risk assessment, risk management, and risk communication, considerations that are all key to comprehensive and effective biosecurity planning. The risk analysis approach should center on specific epidemiological characteristics of the agent, host, and environmental factors affecting reproductive health in the herd (Figure 28.1). A risk analysis for disease control is similar in concept to a hazard analysis and critical control point (HAACCP) system, which helps identify critical control points throughout a production chain where disease incursions may happen and incorporates control measures at each point. Whether a risk analysis or HAACCP system approach is used for protection against reproductive diseases, considerations should involve seeking input from not only producers and veterinarians but possibly also farm employees, family members, economists, and others involved in the daily farm operations.

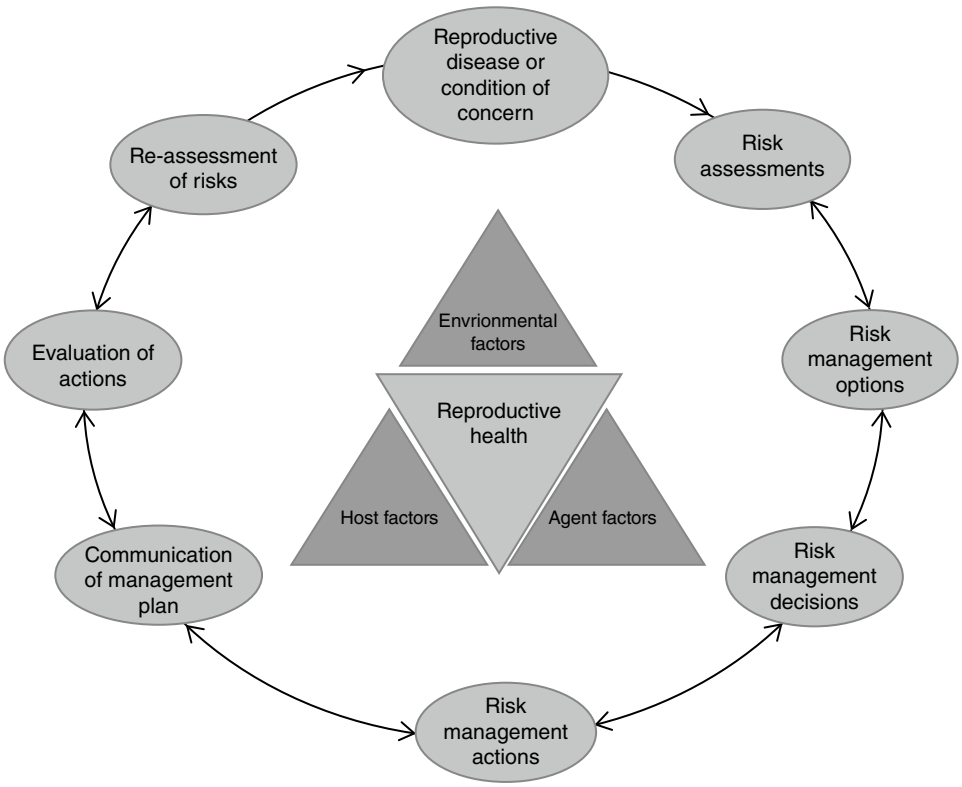


Figure 28.1 The risk analysis approach should center on specific epidemiological characteristics of the agent, host, and environmental factors affecting reproductive health in the herd.

Risk Assessment

It should be recognized in livestock production that every animal in a herd cannot be completely protected against every pathogen affecting reproduction. The purpose of a risk assessment in biosecurity planning for reproductive diseases is to evaluate the true likelihood, impacts, and consequences of pathogen introduction or an outbreak occurring in the herd. This involves hazard identification, exposure assessment, dose–response assessment, and risk characterization. The identification of hazards involves an examination of potential agents involved in reproductive diseases, along with the population at risk or susceptible hosts for disease. A risk assessment should contain estimates of reproductive disease threats, using past and current herd records for production (conception rate, calving rate, calving interval, etc.), and medical history, including vaccination practices and diagnostic testing, where available.

Several infectious agents affecting reproduction should be considered in both beef and dairy herds in the USA, including viruses such as bovine herpesvirus type 1 (BHV-1) and bovine viral diarrhea virus (BVDV), bacteria such as *Brucella abortus* and *Leptospira* spp., and parasites such as *Tritrichomonas foetus* and *Neospora caninum*. Additional agents of concern are dependent on regional and environmental exposures. Host factors for susceptibility to reproductive diseases may be influenced by age, genetics, animal use, vaccination status, nutrition, body condition, and stress. Animals most at risk for reproductive diseases – the breeding females, breeding bulls, and replacement females – can be managed in ways to reduce reproductive disease risks. For example, the use of virgin animals will eliminate the risk of pathogens transmitted venereally such as *T. foetus* and *Campylobacter fetus*.

Other herd management factors, such as animal movements, and environmental conditions must also be considered. It is useful to create a visual map of the farm environment during the initial biosecurity risk assessment to visualize the flow of animal and people traffic throughout the operation. The farm layout should include any buildings, pastures, working areas, feed storage areas, and water sources. Documenting the slope of the ground as well as the location of natural structures such as trees, streams, and ponds can help identify natural barriers for disease control. Nose-to-nose contact points should be identified in indoor as well as outdoor areas, paying special attention to quarantine areas, sick pens, calving pens, and breeding areas. Visitor access points, as well as restricted areas, should be clearly identified. Visualization of these areas and knowledge of associated risks will allow the creation of a flowchart to aid in identifying potential critical control points within an operation as well as identify potential entry points for new pathogens and diseases [5].

Exposure and dose–response assessments determine if the animals at risk are actually exposed to the identified pathogens, how often such exposure occurs, and how much exposure would cause a reproductive health risk to the animals. Environmental factors such as soil type, the presence of natural water sources, pasture management, animal contacts, and housing will have a great impact on pathogen exposure. Risk characterization examines the magnitude of the health risks and how control of those risks should be prioritized. The end result of a properly performed biosecurity risk assessment is a risk determination where animal–pathogen–environment interactions are characterized as high, medium, or low priority for disease.

Risk Management

The risk management stage of a biosecurity risk analysis is often referred to as the "action stage." The purpose of risk management is to establish measurable goals from the risk assessment, determine disease risk reduction strategies using the biosecurity tools that are available, implement biosecurity practices, and evaluate the implemented strategies. Available resources (personnel, equipment, financial), current biosecurity practices, and short- and long-term herd goals must be considered when developing risk management strategies. Goal-setting should include the level of aggressiveness desired and relies on good communication between all parties involved. For example, a less aggressive reproductive goal may be desired by the producer such as cessation of abortions, while a more aggressive goal of a veterinarian might be to establish a herd free from persistent infection by BVD (BVD-PI).

Management techniques to control reproductive diseases include a combination of tools such as movement control, diagnostic test and removal programs, vaccinations, and other enhanced biosecurity measures discussed further in this chapter. Care must be taken to not implement too many changes in an operation at once such that an evaluation of impact cannot be accurately made. Furthermore, producers and employees may become overwhelmed with so many changes that none of them actually become implemented.

Risk Communication

The purpose of risk communication is to translate the results of the risk assessments and summarize risk reduction strategies to an easily understandable form. Documentation of current biosecurity practices and recommended changes will help guide benchmarks for future reproductive goals. Proper communication requires interaction between all parties involved in the initial herd assessment. Additional education of team members and employee training is often

indicated following risk assessment and management recommendations. Standard reproductive herd health protocols such as animal addition protocols, vaccination strategies, and visitor policies should be reviewed and provided in both oral dialogue and written form to have available for future reference.

Communication methods can be very simple, such as a sign hanging in the calving barn, or very complex, such as the development of a statistical process control chart of reproductive efficiency [6]. Regardless of the communication output chosen, it should be readily understandable to the end-user and updated when changes in management practices are made. Written biosecurity risk analyses or HAACCP plans, including treatment protocols and standard operating procedures, will also provide legal documentation if needed. Multilingual materials should be included as appropriate.

While a myriad of biosecurity educational publications are available, most are non-specific and contain wide variations in recommendations [7]. Therefore it is important that biosecurity plans are tailored to farm-specific concerns. Furthermore, it is important to note that a proper risk analysis should be a flexible system, allowing for continuous monitoring, reevaluation, and open communication with all parties involved.

Tools for Intervention: Reducing Effective Animal Contacts

The basic concept behind biosecurity and biocontainment recommendations is that diseases can be prevented or controlled by reducing effective animal contacts. This concept is even truer for reproductive diseases. Effective animal contacts are those contacts made by an uninfected animal that result in infection and disease. Effective animal contacts can be reduced by utilizing a combination of the following concepts:

- Controlling exposure to pathogens
- Reducing animal contacts
- Optimizing an animal or herd's immunity.

Controlling exposure to pathogens can be accomplished by implementing barrier protections such as traffic control and following proper cleaning and disinfection measures. The use of vaccinations and prophylactic medications can complement other pathogen reduction measures by reducing pathogen shedding and exposure. Reducing animal contacts may also involve traffic control and preventing fenceline contacts with outside herds. The use of artificial insemination (AI) and embryo transfer (ET) are two ways to reduce animal contacts in a breeding herd and also prevent exposure to many of the reproductive pathogens of concern. Optimizing animal immunity is accomplished by a variety of methods but should include management strategies such as providing good nutrition, reducing stress, and delivering an appropriate vaccination scheme.

An evaluation of herd production records (including reproductive history, disease incidence, and disease screening tests) will help set goals, identify needs, document actions, and benchmark progress in disease control. However, one of the challenges involved when evaluating the impact of risk reduction strategies in a breeding herd is that results are not often observed until the next calving season. This makes timely record-keeping and monitoring of all health aspects of the herd, such as body condition scores, crucial to the overall health of the herd.

The use of biosecurity practices in beef and dairy herds has been studied and described for various regions and populations across the USA [8, 9]. Management factors such as confinement and pasture utilization are not easily changed, but an awareness of factors predisposing cattle to reproductive diseases under these situations can help make decisions regarding reproductive health more practical. Most biosecurity and biocontainment practices aimed at reducing effective animal contacts will focus on the utilization of movement control, barriers, cleaning and disinfection, and vaccination strategies.

Movement Control

Animal Additions

The biggest risk to reproductive health on a beef or dairy operation is the addition of animals from outside the farm. Animal additions include newly purchased animals as well as animals returning from outside premises such as heifer-rearing facilities and exhibitions. Maintaining a closed herd is an ideal situation where no new animals enter the herd. Unfortunately, this is not always a realistic expectation given the advances in genetics and herd expansions that often need to be made. In general, producers accept that sources of new animal additions, from perceived lowest to highest risk of disease introduction, include private sales, cattle dealers, cattle auctions, and sale barns. Animal additions should only come from herds with a known health status. For example, breeding animal additions should come from farms with effective herd health plans, as demonstrated through herd records, vaccination history, and disease surveillance programs, to minimize the potential for disease introduction. Avoid bringing in non-virgin breeding animals if possible to prevent introduction of venereal diseases. Performing breeding

soundness examinations on all bulls prior to arrival, including trichomoniasis testing on all non-virgin bulls over 12 months of age, will help to identify potentially infectious processes prior to the breeding season.

Quarantine and Isolation

Quarantine refers to the separation of animals exposed to pathogens or at high risk of contracting or carrying pathogens, whereas isolation refers to the separation of animals that are actually sick or known to be infected. Quarantine procedures should be followed whenever animal additions occur. The length of quarantine should cover the incubation period of the most important infectious diseases, generally a minimum of three weeks. Quarantine can be effective in preventing new cases of diseases transmitted by direct contact, such as BVDV, BHV-1, and leptospirosis. However, many diseases, such as neosporosis and BVD-PI, can be present in the subclinical or persistent form and not easily identifiable by visual inspection during this time. In these cases, diagnostic disease testing and vaccinations specific to the operation's herd health program should be performed before the animals are allowed to commingle with the resident herd. This time will also allow the animal to acclimate to the new environment and feed sources.

Dairy herd expansion efforts may pose unique challenges, given space and equipment limitations [10]. The use of a modified receiving pen, with solid-sided walls or double walls, can be used as an alternative quarantine area when separate facilities are not available [5], but strict traffic control and cleaning and disinfection procedures must be followed. If not performed prior to arrival, bull breeding soundness examinations including trichomoniasis and BVD-PI testing should be performed under quarantine in order to detect carriers and to allow ample time to find replacements if needed.

Sick animals should be placed under isolation for effective biocontainment. It is important that the producer understands that the medical treatment of an animal does not equate to cessation of pathogen shedding and that convalescent carriers of disease are possible. Animals that have experienced an abortion or pregnancy loss should be separated from the herd to allow investigation into the cause of the loss, which may include diagnostic testing of the dam or the aborted fetus. Prompt disposal of aborted fetuses, dead calves, and placenta should occur to prevent any transmission by contact or scavenging. Within an operation, animal and personnel movements should occur from the least susceptible animal populations to the most susceptible animals. Sick or high-risk animals, such as those under quarantine, should be handled last whenever possible. In larger facilities, separate personnel may be designated to care for high-risk populations.

Ideal quarantine and isolation facilities should be located as far away from the resident herd as possible – separate and downwind/downstream from the rest of the operation. Water and manure runoff should not be able to flow into common areas or near the resident herd. Separate equipment and protective wear (boots, clothing) should be utilized in quarantine and isolation areas and clearly identified to avoid their use in other animal areas.

Visitor Access

Both farm personnel and visitors can serve as mechanical vectors for reproductive pathogen transmission. This includes neighbors, salespersons, hoof trimmers, AI and ET technicians, and veterinarians. Such persons should be categorized into low-risk, medium-risk, or high-risk individuals based on the number and extent of animal contacts and farm visits made per day. Disease management recommendations can thus be made depending on the individual risk of disease transmission. For example, persons who have contact with animals on multiple farm visits in one day, such as veterinarians and AI technicians, are considered high risk and would be expected to follow more stringent biosecurity protocols. Employees who have their own cattle or have contact with outside animals on a regular basis should also be considered high risk. At a minimum these persons should be expected to have clean clothing and footwear when on the premises and perform stringent cleaning and disinfection before arrival. Lower-risk individuals would be those such as salespersons or equipment mechanics, who should have no contact with animals or animal areas. Visitors should have limited access to the farm, with designated entry points. Signage should be posted restricting visitor access to other areas. A visitor log should be kept and all visitors should be made aware of the farm's biosecurity protocols for visitors, including the required reporting of recent international travel.

Barriers

In addition to movement control, barrier precautions can be used to prevent airborne and contact pathogen transmission between animals. Effective barriers will help decrease unwanted animal contacts by providing an extra layer of protection between infected and susceptible cattle.

Personal Protective Equipment

Personal protective equipment is essential when working with the breeding herd. Obstetric sleeves, gloves, and protective clothing should be used when working with breeding animals, especially when working with potentially infected

uterine fluids, placental tissues, and aborted fetuses. A change of clothing is recommended between farms for persons who make multiple farm visits daily, such as veterinarians and AI technicians. However, this may not always be possible or practical, and guidelines are often made following an appropriate assessment of the true risk of pathogen transmission [11]. For operations with multiple farm locations, separate clothing can be kept at each premises to prevent carrying pathogens from site to site. Protective gear such as disposable coveralls and boots should be provided to visitors who are given access to animal areas if they do not have their own. Whenever possible, used or soiled protective gear should be left on the farm for disposal or cleaning.

Wildlife

Wildlife commonly share ranges and forages with breeding herds and have been shown to be efficient carriers of reproductive pathogens. However, effective physical barriers do not exist to prevent all livestock contact with wildlife. Alternative barriers should be considered to reduce the risks of disease transmission from these populations, which usually occur through the contamination of water and feed.

Wildlife can serve as direct and indirect sources of infectious reproductive pathogens and should be discouraged from feeding and cohabitating with cattle herds. BVDV has been detected in wild ungulates, and deer have experimentally transmitted the virus to naive calves [12]. While the natural transmission of BVDV between deer and cattle has not been demonstrated, the potential should not be overlooked in areas where common feeding occurs. *Brucella abortus*, a pathogen that has eluded national eradication efforts for many years due in part to its persistence in wildlife, has been shown to be transmissible from infected bison to cattle [13, 14]. Wild or feral swine have also become increasingly prevalent in pasture environments. While specific cattle pathogens have yet to be isolated from these swine herds, cases of *Brucella suis* have been reported and remain a concern to the US pork industry [15, 16]. In addition to the potential risks to the reproductive herd, contact with wild ungulates can pose a significant risk to the US livestock population in the event of a foreign animal disease outbreak, such as foot-and-mouth disease. Population control of deer and swine herds may be complicated and should involve collaboration between wildlife management professionals and livestock operators.

Feed and Water Sources

Clean feed and water sources are important for maintaining the health of the breeding herd. *Leptospira* spp. are shed through the urine of infected domestic and wild animals such as deer and other small mammals and are known contaminants of natural water supplies. In operations where multiple water sources are available, pregnant heifers and cows should have access to the cleanest water, avoiding natural streams and ponds. When possible, areas of heavy standing water should not be used for grazing as contaminants such as toxic algae blooms can persist in such conditions. Vaccination schedules may be adjusted to more fully protect the breeding herd against leptospirosis when they are exposed to outside conditions and aversion of natural water supplies is not possible [17].

Storing commodities and feed in an enclosed or protected area helps to prevent fecal contamination with potentially harmful pathogens. Wild canids are the definitive host for *N. caninum*, a parasite causing mid- to late-term abortions in cows (see Chapter 65). Cattle become infected with the parasite when they ingest feed contaminated with feces of infected canids. The parasite has been detected more frequently in dairy cattle populations, where stored feeds are more likely to be ingested. Canines are attracted to feed due to the presence of small rodents such as mice and rates, so rodent control should be routinely practiced. Birds and rodents have also been shown to contaminate feeds by shedding *Salmonella* spp. in their droppings. Birds should be kept from roosting in feeding areas or feed storage areas. Population control of birds and rodents by roost and nesting removal or hazing should be considered. Pesticides should only be used with care. Domestic pets should also be prevented from contaminating livestock feed and water sources.

Farm Environment

Operations may be more at risk to certain pathogens due to their geographical location. Geographical barriers such as streams, wooded areas, tree lines, and ridges, while not easily modified, can be strategically utilized to create barriers between operations or groups of animals. Perimeter control by maintaining good fences is important not only to keep unwanted animals from having contact with the resident herd, but also to keep the resident herd from moving outside the protected area. In addition to maintaining good fences, preventing fenceline contact of the resident herd with other herds is important to prevent nose-to-nose contact and potential transmission of pathogens. Within an operation, high-risk animals should not be allowed to have nose-to-nose contact with the resident herd. If a fenceline must be shared, a temporary fenceline or strand of hotwire can be used to keep animals at least 4 m apart.

The environment in which cattle are housed has a tremendous impact on the risk of reproductive disease development. Beef cattle spend much of their lives on pasture, making it difficult to create effective barriers to other animals and wildlife. An example of proper pasture utilization would be to ensure that weaned or stocker cattle have no fenceline contact with pregnant heifers to reduce the potential for

BVDV transmission. Cattle raised mainly in confinement, as many dairy cattle are, may be more susceptible to pathogens that contaminate feed and water supplies, such as *Neospora caninum* transmitted by canines or *Salmonella* spp. carried by bird and rodent droppings. Most reproductive pathogens such as IBR, *Campylobacter* spp., and *Tritrichomonas* do not survive outside of the animal host for long periods of time. Inclement weather, such as extreme moisture, heat, or drought, can create added stress on a breeding herd, potentially affecting nutrition, decreasing immunity, and increasing susceptibility to disease.

Cleaning and Disinfection

The farm environment is conducive to the survival of many potential bovine pathogens. While the elimination of all debris and manure is impossible, measures can be taken to reduce the pathogen load in the farm environment. Fortunately, many reproductive pathogens are susceptible to common cleaning and disinfection practices. Many excellent resources are available on cleaning and disinfection, and veterinarians and producers should be familiar with the characteristics and limitations of commonly used disinfectants in a farm environment [18].

Equipment and tools used on individual animals should be cleaned and disinfected after each use. Special attention should be given to equipment exposed to animal body fluids such as AI guns, obstetric equipment, and nose leads. Tools such as rakes, shovels, buckets, and halters are ideally not shared between high-risk animals and the resident herd.

Separate machinery or equipment should be used under the following situations: feeding high-risk groups of animals, handling feed and manure, and handling carcasses. However, this is not always possible or practical, and common equipment should be cleaned and disinfected after use in any of these situations. Equipment used off-farm or shared with other operations should also be thoroughly cleaned and disinfected upon return.

The physical removal of debris such as manure and mud from equipment is important to inhibiting pathogen survival. Many viruses and bacteria are susceptible to desiccation and ultraviolet lighting, so the use of chemical disinfectants on large flooring areas or large equipment such as trailers or breeding boxes may not always be indicated in low-risk situations such as daily farm use in a single area. Vehicles of all visitors should be clean of visible mud and manure on arrival. A designated vehicle washing area, which could consist simply of running water, detergents, and disinfectants, should be placed in an area that can be used prior to entry to the farm or animal area. Runoff from the washout area should not have contact with any animal areas.

Quarantine and isolation areas should be thoroughly cleaned and disinfected between groups to avoid cross-contamination in all-in, all-out situations. Where all-in, all-out management of groups is not possible, quarantine, isolation, calving, and other commonly used areas should be scraped and cleaned frequently to avoid manure build-up and the potential for increased pathogen exposure. Placentas should be removed promptly in confinement situations to avoid ingestion by other livestock. Any animal carcasses, including aborted fetuses and tissues, should be removed promptly under any conditions to avoid contact and ingestion by other cattle, wildlife, or domestic pets. Carcass disposal should be performed in compliance with local regulations. In cases of suspected or confirmed reportable animal diseases, specific cleaning and disinfectant measures may be mandated [19].

Feed bunks and water troughs should be cleaned out frequently. Special attention should be paid to waterers that contain lids or floats that may trap debris. BVDV has been shown to survive outside the host for extended periods of time in aqueous materials and in synthetic mucus [20], potentially increasing the risk of transmission through feed and water sources.

Vaccination

Vaccination protocols should be evaluated and updated periodically for optimum timing, type, and antigen selection. Vaccination is one of the most commonly used biosecurity practices by producers and veterinarians. While vaccines rarely prevent infection, proper vaccination strategies may help reduce the incidence or clinical severity of reproductive diseases. Common pathogens targeted by vaccine manufacturers are covered elsewhere in this book and include BHV-1, BVDV, multiple *Leptospira* spp., and *C. fetus* [21]. Uncommonly used vaccines include those against *B. abortus* in females, *N. caninum*, and *T. foetus*. The use of these and other vaccines should be considered only after a thorough biosecurity risk assessment is performed by the producer and the veterinarian to avoid any unnecessary costs. The safety and efficacy of vaccines, including a comparison of the use of modified-live versus killed vaccines, should be determined specific to the animals on the operation. However, the use of vaccines should not be the sole source of protection for a breeding herd and should be used to complement other biosecurity practices.

Diagnostic Disease Testing

Knowledge of a herd's current disease status is important in assessing disease risks and prioritizing control measures. Diagnostic disease testing is a tool that can be used to identify infectious disease risks associated with a single animal or group of animals. Given herd production parameters, statistical probabilities can also be calculated on the

likelihood of disease introduction under specific herd conditions [3]. Diagnostic testing is commonly used to diagnose cases of illness, to augment test and removal programs, to monitor progress in disease control, and to document freedom from disease in prepurchase or postpurchase examinations. Prepurchase testing strategies can be performed prior to bringing new animals onto a premises, and diagnostic disease testing is valuable in detecting subclinically or persistently infected animals, as in the case of trichomoniasis, neosporosis, and BVD-PI. However, in a herd setting it is not always practical to test all incoming animals for all potential reproductive pathogens, and therefore the testing of subsets of animals or pooling of samples may be indicated. As discussed in the section "Vaccination," quarantine of all animal additions will help prevent the spread of any disease while diagnostic test results are pending. Testing for causes of abortion is also important to identify reproductive pathogens that may be present, even though only a small percentage of abortions submitted will actually have a causative agent identified. Diagnostic tests are not perfect and should always be interpreted with characteristics such as test sensitivity and specificity in mind. Sensitivity of a diagnostic test is its ability to correctly identify a positive or diseased animal, and specificity is the ability to correctly identify a negative or non-diseased animal.

While the sensitivity and specificity are characteristics of the diagnostic test itself, predictive values are based on the estimated prevalence of a pathogen or disease, along with sensitivity and specificity to provide a better prediction of the risk of disease in a given population. A positive predictive value estimates the probability of an animal having the pathogen or disease given that the test was positive, and the negative predictive value estimates the probability of the animal being disease-free given that the test was negative. The negative predictive value will have the most importance in prepurchase testing as new owners wish to ensure that imported animals are free from disease. Diagnostic testing schemes are an important part of a herd biosecurity plan but should not be the sole basis for decision-making. The overall health program of the source herd is often more important to consider than a diagnostic test from a single animal. A more complete description of diagnostic testing schemes for the breeding herd can be found in Chapter 43.

Breeding Management

In addition to utilizing the previously described disease intervention tools, a comprehensive reproductive management plan should include having a defined breeding season and performing pregnancy testing in a timely manner. Most beef cattle are managed according to a seasonally defined breeding season, and an outbreak of a reproductive disease may not be recognized until pregnancy checking, often several months after breeding. Reproductive diseases could potentially affect an entire calf crop, and a delay in pregnancy testing could be even more costly. Having all bred females at the same or similar stage of pregnancy can also increase the risk of diseases whose incidence is dependent on the time of infection, such as BVD-PI caused by exposure of the dam from approximately 45 to 125 days of gestation. On the other hand, dairy cattle tend to be bred year around, and changes in reproductive status are likely to be detected sooner.

The purchase, lease, or borrowing of non-virgin bulls for breeding purposes constitutes a significant risk to the reproductive performance of a herd [22]. Trichomoniasis and campylobacteriosis cause decreased reproductive performance in breeding females but do not cause clinical signs in infected males. Breeding soundness examinations of all breeding-aged bulls should be performed prior to purchase and should incorporate trichomoniasis testing. As previously mentioned, states have increasingly been implementing mandatory testing for trichomoniasis for interstate and intrastate movement of animals, and producers and veterinarians should familiarize themselves with their state's regulations. Breeding animals should also be current on vaccinations, including vaccination against BVDV, campylobacteriosis, and leptospirosis. In addition to vaccination, proper external parasite control will help reduce potential transmission of tick-borne diseases which may cause reproductive losses.

AI and ET are effective barriers to the transmission of venereal and other reproductive diseases, while allowing for advancement in genetics and herd expansion. While the production and distribution of semen and embryos pose some risks for pathogen transmission, these are considered to be safe procedures when internationally accepted standards such as those recommended by the Certified Semen Services (CSS) and International Embryo Transfer Society (IETS) are followed [23]. Pathogens such as BVDV and *N. caninum* can cross the placental barrier, so it is important that both donors and recipients are tested free of the disease and properly vaccinated if indicated prior to embryo implantation. Disposable equipment should be used when possible; any items used on multiple animals should be thoroughly cleaned and disinfected between use. Additional information on pathogen control through AI and ET technologies is given in Chapters 81 and 83, respectively.

Special Considerations

Zoonoses

Several reproductive pathogens encountered in beef and dairy operations can be zoonotic [24]. All persons involved in animal handling during the reproductive management of the

herd should take personal protective measures when handling reproductive tissues, fluids, or pharmaceutical products such as hormones. At a minimum, personal protective equipment such as gloves and sleeves should be used and proper cleaning and disinfection procedures followed. While safer strains of modified-live *B. abortus* vaccines are now used, personal protection against iatrogenic infection is still warranted. Reproductive hormones, while not containing pathogens, can be harmful to humans and should be handled with care.

Disaster Preparedness

Beef and dairy cattle producers should include emergency provisions in their biosecurity plan. Unexpected events such as tornadoes, fires, or flooding may require animals to be quickly moved to a different location. Prior to an event, producers are encouraged to identify other producers, sometimes called "sister farms," with similar health programs and biosecurity plans that their animals could be sent to with little risk of disease transmission. Quarantine procedures should be followed on arrival at the sister farm as well as on return to the home farm in order to protect both farms. Animals to be moved should be identified according to a risk assessment, with special consideration given to protecting highly susceptible animals such as pregnant heifers.

In addition to identifying alternative housing, producers should ensure that vaccinations are always current, animals are properly identified, and health records are kept in more than one location. Additional protection against waterborne pathogens such as *Leptospira* spp. may be indicated in flooding situations or events resulting in large amounts of standing water. In emergency situations, the sharing of trailers, loaders, and feed troughs may be unavoidable. If equipment must be shared, cleaning and disinfecting after use and between herds is warranted. Provisions for emergency transportation should be included in preparedness planning. A shelter-in-place system may be most appropriate following certain emergency situations when it may be more harmful to try to move cattle out of a disaster zone. Producers can contact the local county emergency management agency or the state's animal emergency preparedness office for additional disaster planning resources or assistance with emergency sheltering or confinement.

Heightened biosecurity measures may be necessary in the event of an outbreak of a foreign or emerging animal disease. Tools are available to assist beef and dairy producers in developing business continuity plans in the event that animal and product movement restrictions occur in affected areas [25].

Summary

There are many tools available to protect the beef and dairy breeding herd against reproductive pathogens. While taking herd goals into consideration, practical and effective biosecurity plans must be made so that they are acceptable and likely to be followed. Biosecurity and biocontainment plans should be flexible and include disease monitoring and surveillance programs to continually identify operation needs, document management plans, and benchmark progress. A combination of environmental management practices, movement control, barriers, and cleaning and disinfecting protocols can reduce the incidence of reproductive diseases by reducing effective animal contacts. Ultimately, a properly performed risk analysis will assist the veterinarian and livestock producer in developing a comprehensive approach to implementing effective biosecurity practices against reproductive diseases.

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29

Beef Heifer Development

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Introduction

The replacement heifer represents the next generation of genetic progress for the cow herd [1]. Because of the required capital outlay that beef producers invest in these females and the time period required to recoup this investment, it is critical that reproductive efficiency is optimized. In order to get a return on this investment, replacement heifers must become pregnant early in the first breeding season, calve with a minimum of dystocia at two years of age, breed back in a timely fashion, and then continue to be productive for a number of years. Therefore it is critical that replacement females be selected, grown, and managed with these goals in mind.

There has been much discussion within the industry as to the best approach for heifer development. In areas of the USA where grazing land does not compete with crop production, extensive development systems may be advantageous. In this scenario, pasture land is more readily available and less expensive to rent or own. This allows the producer to utilize limited prebreeding selection criteria and expose a much greater number of heifers during the breeding season. Heifers that conceived early in the breeding season are identified and selected at pregnancy examination to fit the replacement needs of the ranch. Heifers that conceived later in the breeding season or remained open are then marketed.

In areas of the Midwest where there is significant influence of crop production on the price and availability of land, more intensive heifer development systems are utilized. Due to limited pasture resources, an aggressive prebreeding selection criterion is needed. This criterion emphasizes early selection, nutritional development, and prebreeding management so that heifers reach puberty prior to the start of the breeding season. The goal of this approach is to limit the number of heifers exposed to only 10–15% more than the number needed to meet replacement needs. Regardless of the system used for development, practitioners need to be involved with these programs to ensure that these females are meeting a set of performance benchmarks for both growth and reproductive targets. This will help ensure optimum reproductive performance, increased female longevity, and increased cow herd profitability.

Replacement Heifer Economics

Table 29.1 summarizes the cost structure of producing a heifer that will join the mature cow herd when she is confirmed pregnant [2]. This budget assumes that the heifer is born in the spring and weaned the following November. She will be bred the following summer and then subsequently calve as a two year old. The single biggest expense associated with the heifer development program is the opportunity cost associated with the market value of the heifer at weaning. This USD $825.46 cost (period 1: conception to weaning) is incurred when the producer decides to keep her in the replacement pool as opposed to selling her at market value.

Costs associated from heifer weaning until the start of the breeding season are primarily associated with the winter feeding program and summer grazing (Table 29.1). Over 85% of this $321 cost is accounted for by feed, labor, yardage, and interest. Dry lot rations or supplementation programs for heifers on forage need to balance feed costs with adequate growth performance. This emphasizes the need for sound nutritional consultation during this time, especially when feed commodity prices are high. The animal health program (including death loss) and grazing costs (pasture rent equivalent) make up the remainder of the inputs during this time frame.

The costs associated with developing replacements through the end of the breeding season are also illustrated

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 29.1 Cost summary (USD) for developing a replacement heifer.

Period 1: Conception to weaning	$852.46
Period 2: Weaning to breeding	$321.00
Period 3: Weaning to pregnancy examination	$129.00
Subtotal	$1275.46
Adjust for 85% heifer conception rate	$1500.00
Adjust for cull heifer credit[a]	$1354.00
Calculated heifer development costs (born 2011)	$529.00

Source: From [2], © 2012, *Beef Magazine*.
[a] Assumes 369-kg heifer at $2.644/kg = $975.60 × 0.15 = $146.34.

in Table 29.1. This $129 estimated cost is primarily associated with grazing and breeding costs. The grazing season is estimated to last four months at $13.60 per month. This budget assumes the purchase of a $4000 bull that will breed 20 heifers per year for a total of four breeding seasons. This breeding cost also considers the annual cost of feeding the bull, interest on the initial investment, and the bull's salvage value when culled. This gives a total investment of $1275 to carry a replacement heifer to approximately 18–20 months of age (pregnancy examination).

Finally, the cost of heifer development must take into account the level of reproductive performance (percent pregnant) and cash value of the open heifer. In this example an 85% pregnancy rate increases the cost of production to $1500 for each pregnant heifer. The income from the sale of open heifers is then credited to the heifer enterprise, bringing the final cost to $1354 per pregnant heifer. This total represents a $529 developmental cash cost being added to the $825.46 opportunity cost incurred at the time of initial selection. With this magnitude of capital outlay, it is critical that these heifers be adequately developed to ensure optimum reproductive performance and longevity.

Selection at Weaning

Recent surveys indicate that approximately 85% of beef replacement heifers were raised on the operation where they calved [3]. Although the majority of herds in the USA utilize some form of individual cow and calf identification, this information is underutilized when it comes to making management decisions. Therefore it is critical that the practitioner help the client understand the importance of accurate data collection as it relates to the heifer development enterprise. Calf birth date, dystocia score or birthweight, female temperament, and calf weaning weight can all be used to make inferences about individual cow productivity over time. This information can then be used to make postweaning selection decisions easier.

The process of heifer development normally begins at the time of weaning with the initial selection of potential replacements. Females should be selected based on age, weight, temperament, and the productivity of their dam. Musculoskeletal soundness must also be evaluated as poor feet and leg structure can have a negative impact on productive longevity [4]. Selecting replacements from calves born in the first half of the calving season will increase the chance of the female reaching puberty by the time the breeding season starts [5–7]. Since the age and breed of the potential replacement are already established by the producer, weaning weight becomes the primary selection parameter and the basis for the developmental program.

Regardless of their birth date, the entire calf crop is typically weaned on the same day, so uniformity of the group of replacements may be an issue. Sorting heifers by weight and frame score is normally the first step in narrowing the pool of replacements at weaning [5]. Economic considerations should be evaluated when there is a wide disparity in heifer weights. Heifers that are too large (frame score) or too light (weight) may be targeted for culling in order to improve the economic efficiency of the replacement program. Beef operations that have a sound replacement development program should only need to retain an additional 10–15% more weaned heifers than they need to become pregnant. This will enable them to maintain current herd size and avoid the opportunity cost of retaining more heifers than needed.

Prebreeding Nutritional Management

Developing replacement heifers to a genetic specific pubertal weight is crucial to the success of the first breeding season and subsequent productivity. Feeding the replacement heifer represents the second highest cost associated with the development program, second only to the opportunity cost of her retention at weaning [8]. Based on a heifer's genetics and expected mature size, a target weight can be selected and the feeding program tailored to meet the needed average daily gain [5–7]. Target weight is simply an estimation of the weight at which individual heifers will reach puberty. Provided we know the weaning weight, target weight, and number of calendar days before the start of the breeding season, rations can be constructed to ensure that heifers reach puberty in a timely fashion. Typically, the rate of gain needed will fall into the range 0.5–0.8 kg/day and represent a total gain of approximately 91 kg for heifers of British breeding.

Traditional intensive developmental programs have emphasized the need for heifers to reach approximately 60–65% of their expected mature weight prior to breeding [5–7]. This practice is utilized to ensure that heifers

reach puberty and are cycling prior to the start of the breeding season. This increases conception rates and the chance of early pregnancies in heifers. Research done at the University of Nebraska-Lincoln has looked at the reproductive performance of heifers that were targeted to reach only 50–55% of their expected mature weight [9–11]. These heifers are overwintered on relatively poor forage (winter range or corn stalks) with supplemental protein. In these studies, reproductive performance was not different between heifers developed to reach 56% (EXT) versus 65% (DL) of their expected mature weight (Table 29.2) [10]. While fewer heifers in the EXT group reached puberty prior to the start of breeding, there was no difference in overall pregnancy rate or percent calving in the first 21 days of the subsequent calving season. However, heifers developed to only 50% of their expected mature weight run the risk of conceiving later in the first breeding season and weaning lighter calves [11]. Reproductive performance in subsequent years was not different among these different heifer groups. Any potential decrease in reproductive performance in these heifers must be weighed against lower feed costs. These cost savings have been documented to range from $22 to $45 per heifer compared with a traditional dry lot development and target weight.

Feeding ionophores to replacement heifers increased feed efficiency and average daily gain, and hastened the onset of puberty [12–14]. An individual daily dose of 200 mg of monensin has been used effectively in these trials. Also, heifers breeding on their third estrus have a higher first-service conception rate (21–36%) compared to those bred at puberty [15, 16]. Therefore gains should be managed so that heifers reach their target weight at least three weeks prior to the beginning of breeding. Selection of the target weight may be based on the average weight of the heifer group or a percentage of the expected mature weight, or extrapolated from the average frame score.

Table 29.2 Effects of dry-lot development (DL) or grazing a combination of corn residue and winter range (EXT) on heifer reproduction.

Trait	DL	EXT	SEM	*P*-value
n, first season	3	3	—	—
Puberty by AI (%)	88	46	0.04	0.01
Conceived to AI, first season (%)	69	58	0.08	0.23
Pregnant to AI, first season (%)	64	54	0.06	0.08
Pregnant, first season (%)	94	92	0.04	0.38
n, second season	2	2	—	—
Pregnant to AI, second season (%)	62	66	0.08	0.61
Final pregnancy rate, second season (%)	93	84	0.14	0.56

Source: From [10], © 2011, American Society of Animal Science.
DL, Heifers consuming a diet in a dry lot post weaning; EXT, heifers supplemented three times per week with the equivalent of 0.45 kg/day 31% crude protein (CP) cube (dry matter [DM] basis) post weaning while grazing corn residue and dormant winter range and subsequently moved to dry lot; AI, artificial insemination.

Prebreeding Selection and Management

Approximately 30–60 days prior to breeding, heifers should undergo a prebreeding evaluation [5, 6, 17]. This is the last step in determining whether or not a heifer will actually enter the breeding program. Heifers should be evaluated on their body weight, reproductive tract score, pelvic dimensions, body condition score, and structural correctness. There is a growing body of evidence that indicates female temperament is highly correlated with that of her calf. Also, feeding performance of the offspring from cows with poor temperament can be negatively affected [18, 19]. There is anecdotal evidence that temperamental heifers have lower conception rates to artificial insemination (AI). At any rate, overly aggressive or easily frightened females should be considered for culling.

When evaluating the reproductive status of the heifer, there is an additional tool that can be used to enhance reproductive tract scoring. The reproductive cycle of the bovine consists of two or three waves of growing and regressing follicles followed by the ovulation of a dominant follicle. These follicular waves consist of secondary follicles that are also known as antral follicles. Antral follicle counts (AFC) can be used to assess reproductive longevity for cows and heifers prior to breeding [20, 21]. This is based on the fact that AFC is correlated with the number of primordial follicles or ovarian reserve. Using an ultrasound unit, each ovary is scanned and the number of antral follicles found on both ovaries is totaled [20]. This method is reliable and reproducible despite variation in genetics, size, stage of estrus, and age of heifers [20]. Research indicates that heifers with low AFC (<15 total follicles) had lower birth weight calves, smaller ovaries, and a decreased pregnancy rate as heifers compared to herdmates with high AFC (>25) [21]. An additional study demonstrated that cows that were repeat breeders had fewer antral follicles in the ovarian cortex compared to normal herdmates [22]. Thus AFC can be utilized in the field to predict fertility and longevity when selecting for replacement heifers prior to breeding.

Other heifers that fail to meet specific selection targets should be culled at this time as well. This evaluation should take place far enough in advance of the breeding season to allow adequate time for needed ration changes if accelerated growth is required. This handling also represents a good opportunity to deliver vaccines and dewormers. This information should be summarized and reported in a format that will justify program decisions to the client.

The health program for the replacement heifer should be targeted against reproductive disease and help to optimize growth rate. This program should complement the suckling calf vaccination protocol that was administered so that protective immunity is maximized. The heifer's first breeding season, first pregnancy, and subsequent reproductive performance should all be considered when defining the timing, delivery, and antigens to include in the vaccination schedule [23]. The history of disease on the ranch, traffic patterns on and off the operation, and previous diagnostic information should also be evaluated when attempting to manage the disease risk.

Vaccines that provide fetal protection against common viral and bacterial diseases should be utilized in these schedules and Beef Quality Assurance (BQA) guidelines should be followed. Deworming schedules can vary greatly depending on rainfall, temperature variation, and forage availability. The class of anthelmintic, level of pasture contamination with infective larvae, and the relative cost of the products will also impact strategic deworming programs [24–26]. Veterinary involvement in these decisions is critical for the successful application of current vaccine and parasite control programs.

Breeding Season Management

Planning for the breeding season should begin early enough to allow time for bull selection (AI or natural service), as well as acquisition and implementation of the estrus synchronization protocol. Evaluation of sires should be based on their ability to pass a breeding soundness examination and their expected progeny difference (EPD) values. Bulls with large scrotal circumferences and prebreeding bull exposure should be utilized, since this will hasten the onset of puberty in their progeny [5, 6]. Progesterone-based synchronization programs do offer the advantage of inducing puberty in some heifers [27]. There are multiple protocols and software programs that can be utilized to manage estrus synchronization [28, 29]. The selected protocols must be individualized for the operation and consider cost, animal handling, and breeding options.

Early pregnancy detection may be a useful management tool to better define the timing of pregnancy, the sire of the calf, and the overall success of the breeding season. This may occur 30–45 days after the end of the AI period or following bull removal [1, 6, 7]. This practice is especially helpful in that it gives an early indication of reproductive failure so that the practitioner can begin the diagnostic work-up much earlier. Early examination of pregnancy may also be used to hasten the culling of non-pregnant or late-bred heifers in the event of forage shortages.

Pregnancy examination provides an opportunity to provide the next step of the health program. Heifers should be dewormed between pregnancy examination and calving, and the health program should center on antigens that cause losses in mid and late gestation [23]. Vaccines for calf scours should be delivered according to label directions as needed. Herd health procedures should follow BQA guidelines and be documented by recording the name, dose, and serial number of the vaccine and the date that it was administered.

Information concerning individual pregnancy status, body condition score, overall pregnancy rate of the group, pregnancy distribution, and culling information should be reported to the client in the form of a written summary [30, 31]. This allows the practitioner and producer to evaluate the strengths and weaknesses of the heifer development enterprise and make needed changes. This type of communication enhances opportunities for proactive planning and fine-tuning of the program. This report will also serve as the planning document to ensure that the pregnant heifers are well managed all the way through the subsequent calving season.

Postbreeding Through Calving

Once heifers are determined to be pregnant, they need to be managed to calve in moderate body condition (average condition score 5.5–6.5). Heifers calving in moderate condition usually have less dystocia, a shorter postpartum interval, and higher pregnancy rates after their second breeding season. Nutrition prior to parturition can also affect postnatal development of the calf by influencing colostrum quality and quantity. Calves produced from first-calf heifers have decreased serum immunoglobulins at 24 hours of age compared to multiparous dams [32]. This is likely due to a decrease in volume and not necessarily the overall quality of the colostrum. A previous study showed calves born to two-year-old heifers in poor body condition exhibited less vigor and lower serum immunoglobulin concentrations at 24 hours of age [33]. Postnatal development in relationship to colostrum intake needs to be considered as it can directly impact the morbidity and mortality of future replacement heifers.

Following pregnancy examination, weight gains need to focus on having the heifers reach approximately 85% of their expected mature weight by the time they calve. Moderate daily gains in the range of 0.5 kg/day will usually be adequate to ensure that heifers reach their first calving with adequate frame, pelvis, and body condition. It is useful to remember that approximately 70–75% of fetal growth occurs during the last trimester of pregnancy and rations should be adjusted accordingly [7, 12]. These adjustments need to include adequate protein, energy, and trace minerals. This is especially important for the first three months after calving, if heifers are expected to be able to lactate, grow, and rebreed in a timely fashion [34, 35].

It could be argued that future replacement heifer development begins while that individual heifer is in utero. The maternal environment can have a great impact on subsequent productivity even after the calf is born. Studies have reported instances of compromised maternal nutrition during gestation resulting in neonatal mortality, intestinal and respiratory dysfunction, metabolic disorders, decreased postnatal growth rates, and reduced meat quality [36–38]. This phenomenon has been referred to as developmental or fetal programming. Nutritional shortages may have a negative impact on the developing fetus at all stages of pregnancy, but this can be especially severe during the last trimester.

Calves born to protein-restricted cows will show reduced feeding and reproductive performance [38]. Heifer calves from these cows had lower body weights at weaning, prebreeding, and pregnancy examination. Contemporary heifers from protein-supplemented cows reached puberty at an earlier age and had higher pregnancy rates after their first breeding season. This resulted in a 28% increase in the proportion of heifers calving in the first 21 days of the calving season. The steer mates to these heifers also showed an improvement in weaning weight, feedyard dry matter intake, percent grading choice, and marbling score [38]. Table 29.3 illustrates two studies that evaluated the effect of maternal protein supplementation on heifer progeny reproductive performance [38]. Heifers from supplemented cows exhibited improved weaning weight, growth, and pregnancy rate. A similar effect was observed in another study that utilized distillers grains to increase the energy content of a gestating cow's diet [39]. In this trial, cow supplementation resulted in an increase in heifer progeny conception rate to AI, even though there was no change in AFC or age at puberty. Although the mechanisms by which placental and fetal programming occur are not clear, managing resources to ensure proper female nutrition during critical points of gestation can improve calf performance, calf health, and future reproductive potential. In those cases of poor reproductive performance in replacement heifers, the nutritional management of the *entire* cow herd must be included as part of the diagnostic work-up.

Table 29.3 Effect of maternal protein supplementation on heifer progeny performance.

	Dietary treatment			
	Martin et al. [37][a]		Funston et al. [32][b]	
	NS	SUP	NS	SUP
Weaning BW (lb)	456	467	492[c]	511[d]
Adj. 205-d wt (lb)	481[c]	498[d]	470	478
DMI (lb/day)	14.39	14.88	20.89	20.50
ADG (lb/day)	0.90	0.88	1.86[e]	1.74[f]
Residual feed intake	−0.12	0.07	0.08	−0.04
Age at puberty (days)	334	339	365[e]	352[f]
Pregnant (%)	80[c]	93[d]	83	90

BW, Birth weight; Adj. 205-d wt, adjusted 205-day weight; DMI, dry matter intake; ADG, average daily gain.

[a] NS, Dams did not receive protein supplement while grazing dormant Sandhills range during the last third of gestation; SUP, dams were supplemented three times per week with the equivalent of 1.0 lb/day of 42% CP cube (DM basis) while grazing dormant Sandhills range during the last third of gestation.

[b] NS, Dams did not receive protein supplement while grazing dormant Sandhills range or corn residue during the last third of gestation; SUP, dams were supplemented three times per week with the equivalent of 1.0 lb/day of a 28% CP cube (DM basis) while grazing dormant Sandhills range or corn residue during the last third of gestation.

[c,d] Means within a study with different superscripts differ ($P \leq 0.05$).

[e,f] Means within a study with different superscripts differ ($P \leq 0.10$).

Summary

Replacement heifers represent a relatively large capital investment on the part of the producer. Therefore they need to be managed appropriately to ensure that they breed, calve, and rebreed in a timely fashion. Events crucial to heifer development center on proper selection, nutritional management, breeding season management, and program evaluation. Careful monitoring of heifer growth and performance at these critical times is an important component of successful development. Record systems should be created and utilized so that the replacement heifer program can be evaluated and benchmarked objectively over time. Practitioners are in a unique position to help their clients assess this part of their operation and make needed changes.

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30

Dairy Heifer Development from Weaning to Calving

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Introduction

After weaning the replacement dairy heifer, one must have fully knowledge of the impact caused by dietary and health management associated with the task to raise a heifer that is efficient and profitable. It is of common understanding that performance, production, and reproduction during first lactation is the best indicator that a successful rearing program has been used.

Veterinary practitioners must educate producers about three basic ground rules: (1) the producer should never underestimate the costs associated with heifer rearing programs – roughly 25% of a dairy farm's production costs are allocated to replacement heifers, and of this 25%, feed is responsible for about 50% of the total cost of raising dairy heifers [1]; (2) excessive or too steep a rise in weight gain prior to puberty is known to directly negatively affect reproduction and production at first lactation; and (3) age at first calving should be around 22–24 months, with an optimal body weight; this is critical to minimize feed costs and increase productive life.

The Weaned Heifer

There are many alternatives with regard to housing and delivery of feed to recently weaned heifers. From pasture to confinement, it is imperative to avoid overcrowding and to move heifers out of the hutches in small groups of 5–10 head at a time [2, 3]. This is when the heifers experience the most stressful time as a group. The main advantage of having a small group is that this allows the heifers the opportunity to adjust to diet change, pathogen challenges, and proper growth. Ultimately, the goal is to raise healthy heifers that reach the breeding group as homogeneous and balanced as possible; however, it is imperative to keep in mind that when calves are grouped, the risk of diseases such as respiratory conditions and diarrhea, specifically coccidiosis in this age group (>30 days old), increases.

Body weight is directly correlated to puberty as well as first-lactation milk yield. An easy rule of thumb to remember is that target body weight at initiation of breeding should be around 55% of mature body weight and 82% of mature body weight at calving. It is important to stress that growth rate is by far the major contributory factor to a successful breeding heifer program [4]. Monitoring body condition (weight gain) provides valuable field information with regard to weight changes. One body condition score equals 54 kg of body weight [5]. Table 30.1 summarizes the ideal body condition score at different production stages.

Heifers express their first estrus at 9–12 months of age and weight of approximately 280–300 kg [3]. The identification of heifers that are suitable to be bred is essential. Individuals not reproductively sound, underdeveloped, or freemartins should be removed as soon as possible. Therefore it is recommended to move heifers into the breeding pool two to three months prior to expected initiation of breeding, so estrus and potential problems can be identified. The diet offered should provide a stable rate of gain, since excessive growth at this point is not necessary and wasteful. Heifers that are within two months from calving, often referred as "springing heifers," should be fed ingredients similar to the lactating group, with good-quality forages and a limited amount of concentrate (1–2% of body weight), with the intention of a smooth transition, which in turn supports a high dry matter intake (DMI) immediately after calving and optimum peak milk. Close attention should be paid to the sodium and potassium concentration in the diet as it is known to be one of the causes of udder edema after calving [6].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 30.1 Target body condition score and production cycle.

	Body condition score (1–5 scoring system)
Cows	
Calving	3.5
Peak milk	2.75
Mid lactation	3.0
Drying off	3.5
Replacement heifers	
Six months old	2.75
Breeding age	3.0
Calving	3.5

The accurate identification and removal of extra teats (if more than four teats are present in the mammary gland) is a procedure practitioners and producers routinely perform on farm and is of great importance for the health of the dairy heifer and her future performance, as it is common knowledge that the presence of extra teats on lactating cows interferes with milking procedures, especially on dairy farms that use robotic milkers, and increases the odds of subclinical and clinical mastitis. The most recent National Animal Health Monitoring System (NAHMS) Dairy 2014 survey reported that roughly 47% of dairy operations routinely remove extra teats. The same survey also reported a variation among regions (west versus east) when it comes to the age of the heifer, but short of 45% of operations routinely remove extra teats when the heifer calves are less than 12 weeks [7]. The author recommends the identification of extra teats and their removal using a sharp, clean Metzenbaum or Mayo pair of scissors at around weaning time (10–12 weeks of age). It is imperative that the producer and/or the practitioner observes a strict biosecurity protocol by utilizing cold sterilization of the scissors between animals to avoid the transmission of blood-borne diseases such as anaplasmosis, bovine viral diarrhea virus, and bovine leukemia virus.

A summary of the optimum target body weight and height as well as average daily gain for different age groups of Holsteins and Jerseys is provided in Table 30.2. It is important to note that these are typical guidelines to help practitioners and producers; however, these values may vary according to region and type of system used to raise replacement heifers.

Health events are undoubtedly a major driving factor that increases the costs of raising replacement heifers due to poor performance and/or extra labor associated with medical treatments, premature culling, or death loss. Protozoal (*Eimeria* sp.) diarrhea followed by respiratory diseases are the main causes of postweaning sickness. Ionophore antibiotics do not currently require a Veterinary Feed Directive (VFD) prescription; however, it is the veterinarian's and/or nutritionist's responsibility to always advise the producer to follow label directions. In this case, ionophores not only aid in preventing protozoal diarrhea (coccidiosis), but also increase feed efficiency by reducing methane losses and increasing propionate production in the rumen. Propionate is essential for gluconeogenesis in the liver and therefore energy produced. This class of antibiotics is widely used in beef-feedlot cattle and in the early 2000s a label for lactating dairy cattle was established. Table 30.3 summarizes the most common coccidiostats used in bovine medicine. Duffield and Bagg [8] published a review that addresses the use of monensin in lactating dairy cattle. Diets containing monensin when offered before calving have positive effects on energy metabolism and conversion in early lactation, and its use is associated with a reduction in subclinical ketosis and postpartum diseases. Despite the fact that 1 kg/day of milk production may be gained when monensin is used in early lactation, depression of milk fat components may occur with the use of ionophores.

Milk fat depression may be seen when lasalocid, specifically, is used, due to its effect on volatile fatty acid production in the rumen; however, this adverse effect is known to be dose dependent [9]. As per the label, a dose of monensin in calves to aid in the prevention and control of coccidiosis, as well as improve feed efficiency and therefore increase the rate of weight gain, must not exceed 200 mg/day per head [7].

Sulfonamides are also used to treat episodes of diarrhea cause by coccidians. Sulfadimethoxine and sulfamethazine are the only sulfa products labeled for use in food animals; all others are prohibited under the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994 [10]. Sulfamethazine is available in sustained-release bolus for oral administration in non-lactating dairy cattle, and when used according to label directions provides therapeutic levels for up to 72 hours. If the intent is to treat lactating dairy cows, sulfadimethoxine is the only sulfa drug available. Intravenous and oral routes exist for this antimicrobial and label recommendations should be followed with respect to indication, dosage, and frequency of administration. Most importantly, a withdrawal period of seven days for meat and withholding period of 60 hours for milk must be observed.

Another important health condition is with respect to the incidence of external and/or internal parasites. Internal parasitism directly affects heifer development due to the decrease in rate of weight gain by decreasing the ability of the gastrointestinal tract to metabolize and absorb nutrients. Similarly, external parasites such as ticks, mosquitos, stable flies, house flies, horn flies, horse flies, deer flies, and lice may feed from the host's blood, skin, and/or hair, causing

Table 30.2 Summary of target body weight, height, and average daily gain (ADG) for different age groups of Holstein and Jersey heifers.

Age (months)	Weight (kg)		Height (cm)		ADG (kg/day)
	Holstein	Jersey	Holstein	Jersey	
6	160	113	100	90	0.7–0.8
12	295	204	119	107	0.7–0.8
18	408	272	127	114	(Post puberty) 0.6–0.7
24	544	363	132	122	(Post puberty) 0.6–0.7

Sources: [3, 5].

Table 30.3 Summary of coccidiostats used in bovine medicine.

Active drug	Brand name (commonly used)	Prevention	Treatment
Amprolium	Corid®	5 mg/kg for 28 days	10 mg/kg for 5 days
Sulfamethazine[a]	Sustain III bolus®	N/A	32.1 g/200 lb once
Sulfadimethoxine[b]	Albon®	N/A	25 mg/lb followed by 12.5 mg/lb for 3–4 days
Lasalocid	Deccox®	0.5 mg/kg for 28 days	N/A
Decoquinate	Bovatec®	1.0 mg/kg for 28 days	N/A
Monensin	Rumensin®	1.2 mg/kg for 28 days	N/A

Note: Always read and follow label instructions. Consult the veterinarian for recommendations and establishment of proper withdrawal times for the production animal in question to ensure AMDUCA is observed.
[a]Sustained release bolus providing therapy for up to 72 hours. For use only in beef and female dairy cattle less than 20 months of age. Do not give to calves used for veal.
[b]Approved for use in lactating dairy cows. Milk withdrawal is 60 hours and meat withdrawal is 7 days.

severe skin conditions and even open wounds, which leads to pain, discomfort, and overall aggravation. Outbreaks tend to be seasonal and occurrence depends on the region, so treatment and control methods must be implemented accordingly. Additionally, lice and mites are a significant problem due to their potential capability as vectors, through vertical and horizontal transmission [11]. Flies are potentially one of the contributors to the increase in bacterial counts in milk due to a non-hygienic environment and are a major vector in the transmission of infectious bovine keratoconjunctivitis and certain blood-borne diseases [12].

McGuirk [13] summarizes the important points to be analyzed when investigating a respiratory outbreak. Morbidity and mortality data, vaccination and treatment protocols, nutrition, age, housing condition, and case definition are some of the records needed. These criteria facilitate the development of protocols designed to improve early detection of disease and, if indicated, the initiation of an effective treatment.

According to the NAHMS Dairy 2014 survey, respiratory disease accounts for roughly 59% of weaned heifer deaths. Of those diagnosed with respiratory disease, over 90% of the weaned heifers were treated with antimicrobials. Florfenicols, macrolides, and tetracyclines in that order are the top three antimicrobials used by the majority of dairy operations, according to NAHMS [7].

Prebreeding vaccination is imperative. As described in the companion chapter to this on developing beef heifers (Chapter 29), a booster of common core vaccines 30–45 days prior to breeding is strongly recommended. Although there is variability among farms and populations at risk, the general consensus is that ideally replacement heifers must receive a modified-live respiratory five-way vaccine (IBR, PI3, BRSV, BVDV type 1 and 2) before weaning and additional co-mingling, as well as pentavalent *Leptospira* serovars (*Pomona*, *Grippotyphosa*, *Canicola*, *Icterohaemorrhagiae*, and *Hardjo*) at breeding time. This provides the opportunity to acquire optimum protection. Always follow the label directions, especially with respect to the usage of a modified live vaccine product in pregnant cattle. Remember that pregnant cattle may only be boostered with a modified live product if that individual had been exposed to that same product within the past 12 months; also, if there is a high incidence of respiratory

disease in preweaned calves, it is crucial to evaluate the colostrum management program and lactating/dry cow vaccination protocols.

Several antimicrobials labeled for treatment of respiratory disease are available in the USA (Table 30.4). Dosage, frequency, route of administration, spectrum of activity, and meat withdrawal periods vary. One must consider that most of them have specific label directions with respect to age group allowed to be treated; if extra-label drug use is practiced, some of these products may or may not fall under the prohibited drug category of AMDUCA (e.g. fluoroquinolones and certain sulfonamides) [10].

Subclinical and clinical mastitis has been associated with milk and reproductive losses of first-calf heifers [14]. Intramammary (IMM) antimicrobial products are often used in springing heifers with the objective of decreasing the prevalence of IMM infection after parturition, reducing somatic cell counts, and potentially increasing milk production. A review of mastitis treatment published by Royster and Wagoner in 2015 [15] summarized all the available IMM treatments and stated that the evidence has shown that IMM treatments of springing heifers reduce the prevalence of intramammary infections (IMI) at calving; however, little evidence is available that actually supports the advantage of instituting this treatment protocol as it is neither economical nor practical unless there is a high risk of infection or the farm lacks other specific management programs/protocols.

The use of IMM antimicrobials in springing heifers is considered extra-label drug use; the treatment of heifers before calving should only be recommended and prescribed by a licensed veterinarian and in herds that have demonstrated high incidence of heifer mastitis [16, 17]. In 2012, de Vliegher et al. published a comprehensive review about mastitis in dairy heifers. The group concluded that often non-lactating heifers are infected with major mastitis-causing pathogens and there is potentially horizontal transmission. Prevention is the key; overall hygiene, especially at calving, fly control, and the prevention of cross-suckling are some of the recommendations. These authors also reported that, in general, coagulase-negative staphylococci are known to be the principal etiologic agent, followed by coagulase-positive staphylococci and environmental pathogens [17]. Therefore, when working with herds with a high incidence of heifer mastitis the cause must be identified and the specific risk factors for such analyzed. The IMM use of antimicrobials in non-lactating heifers has not always proven efficacious when it comes to improvements in overall udder health and milk yield; however, the combination of IMM antimicrobials and the use of a teat sealant has been shown to decrease the incidence of clinical and subclinical mastitis during the first nine months of lactation and have a positive long-term effect on the linear somatic cell score [18]. Table 30.5 summarizes the top four IMM antimicrobials commonly used in springing heifers. As mentioned above, it is considered extra label drug use and must be prescribed by a licensed veterinarian with a valid client patient relationship (VCPR) on record according to AMDUCA in the USA.

For welfare and biosecurity reasons, prompt identification of a sick yearling heifer is crucial; however, the author strongly stresses the importance of addressing the potential problem in a herd health manner. Treating

Table 30.4 Summary of most commonly used labeled antimicrobials available in the USA for treatment of respiratory disease in dairy cattle.

Drug	Product	Dose	Route of administration	Duration	Dairy older than 20 months of age	Milk/meat withdrawal
Oxytetracycline	200 mg/ml LA200®	4.5 ml/45 kg	Subcutaneously	48–72 hours	Yes	96 hours/28 days
Ceftiofur (one treatment)	Exceed®	1.5 ml/45 kg	Subcutaneously in base of ear	7 days	Yes (prohibited ELDU)	Zero/13 days
Tulathromycin	Draxxin®	1.1 ml/45 kg	Subcutaneously	10–14 days	No (ELDU)	N/A/18 days
Tilmicosin	Micotil®	1.5 ml/45 kg	Subcutaneously	48 hours	No (ELDU)	N/A42 days
Florfenicol	NuFlor®	3 ml/45 kg 6 ml/45 kg (single) treatment	Intramuscularly Subcutaneously (one-time dose)	48 hours 4 days	No (ELDU)	N/A/28 days (i.m.) N/A/38 days (s.c.)
Gamithromycin	Zactran®	2 ml/50 kg	Subcutaneously	10 days	No (ELDU)	N/A/35 days
Enrofloxin	Baytril®	1.1–2.3 ml/45 kg or 3.4–5.7 ml/45 kg	Subcutaneously	24 hours Single dose	Prohibited by FDA	N/A/28 days
Tildipirosin	Zuprevo®	1 ml/45 kg	Subcutaneously	Single dose	No (ELDU)	N/A/4 days

ELDU, Extra-label drug use; FDA, Food and Drug Administration; N/A, non-applicable.

Table 30.5 Summary of the top four dry cow therapy intramammary antimicrobials most commonly used in springing heifers.

Name	Product	Indication per label	Milk/meat withdrawal	Minimum dry period length
Cephapirin benzathine 300 mg	Cefa-dri® or Tomorrow®	*S. agalactiae*, *S. aureus*, including penicillin-resistant strains	72 hours/42 days	30 days
Ceftiofur hydrochloride 500 mg	Spectramast DC®	*S. aureus*, *S. dysgalactiae*, *S. uberis*	No milk discard/16 days	30 days
Procaine penicillin G + dihydrostreptomycin (1 000 000 IU/1 g)	Quartermaster®	*S. aureus*	96 hours/60 days	42 days
Cloxacillin benzathine 500 mg	Dry-Clox®	Gram-positive bacteria including *S. agalactiae*, penicillin-resistant and non-penicillinase-producing *S. aureus*	No milk discard/30 days	30 days

individual animals is important but does not solve the problem. On-farm monitoring and preventative methods are vital. This information allows the analysis of how efficacious the management program is in preventing the occurrence and transmission of the disease or condition in question.

The National Research Council [19] suggests an average daily gain of 0.7–0.8 kg, assuming that a healthy calf is being raised in a thermoneutral environment. In addition, Drackley [4] clearly states that a growth rate of 0.9 kg/day is achievable and does not affect mammary development, but needs to be adjusted to improve animal health and farm productivity. Therefore DMI must be managed properly based on environmental conditions. DMI should be increased when temperatures are below 15 °C; failure to provide extra energy can result in decreases of up to 0.2 kg/day of average daily gain [3]. If forage availability is not an issue, heifers raised on pasture should not have problems reaching the goal suggested by the National Research Council [19], despite the extreme variability in quantity and quality of the feedstuff offered. Special attention should be paid to the trace and major minerals, as supplementation may be warranted depending on the region and season.

In addition to the heifer grower ration, early-cut forages are preferred for the recently weaned younger heifers, as they are higher in energy and protein and lower in fiber. High-fiber products should be avoided in young calves as the rumen–reticulum is still under development and unable to fully digest fiber-rich diets [2]. The major goal when it comes to feeding is to sustain uniform weight gain and growth, which will allow the heifer calf to attain puberty and thus breeding at the recommended age.

Summary

In summary, a successful heifer rearing program consists of a consecutive series of systems employed to ultimately raise a heifer with minimum expense to be reproductively efficient and to deliver an excellent productive first lactation. Nutrition is known to be one of the most important factors at this stage of life, as it is directly correlated to reproduction, growth, and overall health.

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Nutrition and Reproduction in the Beef Cow

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Introduction

Nutrient requirements of beef cows vary in a predictable manner relative to the timing of breeding, weaning, and calving. Protein, energy, vitamin, and mineral requirements reach a zenith four to eight weeks postpartum, coincident with peak milk production. This is followed by a normal attenuation in nutrient requirements as milk production wanes; shortly after weaning takes place, nutrient requirements reach their annual nadir. It is not until the final 60–80 days of gestation that they begin to rise again to support exponential fetal growth and accelerated uterine metabolism [1]. Managers of beef cows and heifers are largely in control of the timing of these events; they decide when breeding exposure begins and, therefore, when subsequent calving will take place (i.e. an average of 282.5 ± 18 days following conception) [2]. Managers also determine when weaning will take place, bringing a halt to lactation and setting bounds for the net nutrient demands associated with milk production.

In spite of the predictability of beef cow nutrient requirements, provision of a cost-effective diet that meets animal requirements during each day of a 12-month production cycle can be challenging. When elevated nutrient demand coincides with poor diet quality, body energy and protein reserves will be mobilized to support metabolic processes with a presumed hierarchy. Basal metabolism is prioritized first, followed in descending order by: (i) motion necessary to gather food, (ii) growth, (iii) minimum energy reserves, (iv) maintenance of an existing pregnancy, (v) maintenance of a live offspring via lactation, (vi) secondary energy reserves, (vii) reproductive function including conception, and (viii) tertiary energy reserves [3].

Ironically, the most important feature of this hierarchy to long-term profitability of the beef cow, timely conception, is prioritized next-to-last. Nutritional shortfalls that occur during late gestation and early lactation, which immediately precede breeding exposure, result in mobilization of body reserves. Should mobilization of body reserves be significant during these times, either delayed conception or failure to conceive will be the likely result [4]. The former represents a loss of gross income because the resulting offspring is likely to be younger and lighter than its contemporaries at any given marketing date. The latter represents a loss of gross income because the affected female is usually sold, bringing a premature end to her earning potential.

Under the best of circumstances, lifetime net income derived from a beef cow is modest. Therefore care must be taken to strategically build body energy and protein reserves at a time during the production cycle when high-priority metabolic functions (i.e. late gestation and lactation) are not taking place. This minimizes the need for concentrate feeding and allows the beef cow to meet the majority of her nutrient needs with grazed forages and roughages.

This chapter focuses on strategic management of the 12-month cow-calf production cycle to leverage economic and biological efficiencies to support body energy and protein reserves of breeding females. Related issues such as heifer development, bull nutrition, fetal programming, and certain specific nutritional challenges will also be addressed.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

The 12-Month Cow-Calf Production Cycle – The Driver of Nutrient Requirements

To begin any discussion of the cyclic nature of nutrient requirements in the beef cow, it becomes necessary to define how nutrient requirements vary with the physiological state of the animal. For practical purposes, 12 months are required for a breeding female to become pregnant, to carry a fetus to term, to give birth, to begin lactation, and to become pregnant again. At some point during this 12-month period, a live offspring will be weaned and lactation will come to an abrupt halt. Each of these physiologically significant events influences energy, protein, vitamin, and mineral requirements, either suddenly (as with birth and weaning) or over a continuum (as with gestation and lactation).

A 12-month interval between conceptions is effectively a biological maximum for breeding beef females, multiple births notwithstanding. It is possible but rare for cows to have calving intervals that are significantly less than 12 months, whereas cows with calving intervals greater than 12 months are at risk of being culled from the herd because they fail to conceive in a timely manner.

Table 31.1 provides an overview of a 12-month production cycle for a Hereford × Angus cow with a live weight of 589.6 kg (i.e. 1300 lb). It highlights the elasticity in requirements for net energy, metabolizable protein, calcium, and phosphorus needed to support maintenance, pregnancy, and lactation [3, 5]. The calculations in Table 31.1 represent minimum nutrient needs for a cow of specified shrunk body weight (i.e. live weight × 0.96), milk-production potential (i.e. 9.75 kg/day peak), milk composition, lactation duration (i.e. 210 days), and calf birth weight (36.3 kg). They assume thermoneutral conditions, confinement feeding, a dry and mud-free hair coat, no feeding of ionophores or non-ionophore antibiotics, and no fluctuation in cow body weight per se. Any changes in these assumptions can be expected to influence nutrient requirements in significant ways.

The value of reviewing a comprehensive summary of beef-cow nutrient requirements such as that found in Table 31.1 lies in developing an appreciation for the magnitude of change in nutrient requirements needed to support milk production, fetal growth, and uterine development. For example, the net-energy-for-maintenance (NEm) requirement of the cow in the example (i.e. 8.94 Mcal/day) was predicted to increase by over 78% at peak lactation (i.e. 15.94 Mcal/day). In addition, the extremely rapid growth of the fetus during the final month of gestation was predicted to result in a weight gain of 1.54 kg/day (nearly 3.4 lb) to the combined cow, gravid uterus, and fetus. Nearly 40% of that increase in mass was attributable to the gravid uterus alone; the combined gravid uterus and fetus were predicted to weigh approximately 60 kg during the last month of pregnancy, whereas calf birth weight was assumed to be only 36.3 kg.

The fact that these changes in nutrient requirements occur predictably relative to the timing of breeding, calving, and weaning dictates that managers of beef cattle not only be aware of when they occur but also must be capable of suppling additional nutrients in targeted amounts when needed. Managers must also be aware that significant locomotion, thermal stress, and degradation of hair-coat condition can compound the difficulties associated with maintaining reproductively adequate body reserves of protein and energy.

Body Condition Scoring – the Starting Point for Nutritional Management

Humans perceive the protein and energy reserves of beef cattle as *body condition*. Body condition is a collective term describing the relative amount of muscle and subcutaneous fat covering skeletal features. In general, timely achievement of moderate body condition allows optimal reproductive function, including minimized dystocia, minimized length of postpartum anestrus, and early rebreeding. Thin body condition at calving has been associated with dystocia and subsequent reproductive failure, whereas fat body condition at calving has been associated with poor feed intake, dystocia, metabolic disease, and excessive maintenance costs [6–10].

Body condition is generally quantified as body condition score (BCS). The most commonly applied body condition scoring systems use a nine-point scale, where BCS 1 is extremely emaciated and BCS 9 is extremely obese [11–13]. Optimum body condition is generally thought to be equivalent to a BCS score of 5 or 6. Estimated empty-body fat and protein percentages associated with each condition score are depicted in Table 31.2 [3].

Quantitative management of weight change in growing and finishing beef cattle is uncomplicated. If a manager knows current animal body weight and sets a goal for final body weight, a diet can usually be formulated that will allow the target body weight to be reached at a specified point in time. Unfortunately, assets needed for quantitative management of nutrition (e.g. animal scales, feed scales, diet formulation software, and detailed knowledge of nutritional chemistry) are fairly rare on cow-calf operations in the USA.

Table 31.1 Nutrient requirements of beef cows: effects of physiological state and time[a].

Mature body weight, kg	589.6	Calf birth weight, kg	36.3	Peak milk, kg/day	9.75	Milk fat, %	4.0	
Shrunk body weight, kg	566.0	Calf age at weaning, weeks	30	Time of peak milk, days	60	Milk protein, %	3.4	
Cow age, months	60	Conception postpartum, days	66	Lactation duration, days	210	Milk SNF, %	8.3	

	Months since calving											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Item	**Net energy for maintenance required, Mcal/day**											
Maintenance	8.94	8.94	8.94	8.94	8.94	8.94	8.94	8.94	8.94	8.94	8.94	8.94
Pregnancy	0.00	0.00	0.01	0.03	0.10	0.20	0.30	0.70	1.20	2.10	3.60	5.60
Lactation	5.83	7.00	6.30	5.04	3.78	2.72	1.80	0.00	0.00	0.00	0.00	0.00
Total	14.77	15.94	15.25	14.01	12.82	11.86	11.04	9.64	10.14	11.04	12.54	14.54
	Metabolizable protein required, g/day											
Maintenance	441	441	441	441	441	441	441	441	441	441	441	441
Pregnancy	0	0	1	2	3	7	13	25	46	81	141	232
Lactation	425	510	459	367	275	198	131	0	0	0	0	0
Total	866	951	901	810	719	646	585	466	487	522	582	673
	Calcium required, g/day											
Maintenance	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4
Pregnancy	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.3	11.3	11.3	11.3
Lactation	20.0	24.0	22.0	17.0	13.0	9.0	5.9	0.0	0.0	0.0	0.0	0.0
Total	37.4	41.4	39.4	34.4	30.4	26.4	23.3	17.4	28.7	28.7	28.7	28.7
	Phosphorus required, g/day											
Maintenance	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3
Pregnancy	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.6	4.6	4.6	4.6
Lactation	12.0	14.0	12.0	10.0	7.0	5.0	3.3	0.0	0.0	0.0	0.0	0.0
Total	25.3	27.3	25.3	23.3	20.3	18.3	16.6	13.3	17.9	17.9	17.9	17.9
	Milk production and gravid uterus + fetal gain, kg/day											
Milk	8.12	9.75	8.75	7.03	5.26	3.81	2.51	0.00	0.00	0.00	0.00	0.00
Pregnancy	0.00	0.00	0.03	0.06	0.09	0.15	0.24	0.38	0.56	0.81	1.17	1.54
	Estimated tissue mass, kg											
Shrunk body weight	566	566	566	566	566	566	566	566	566	566	566	566
Gravid uterus + fetus	0	0	1	1	2	4	7	11	18	27	41	60
Total	566	566	567	567	568	570	573	577	584	593	607	626

Sources: [3, 5].

[a] Calculations assume thermoneutral conditions, confinement feeding, dry hair coat, no feed additives, and Hereford × Angus breeding.

Table 31.2 Estimated empty-body fat and protein associated with BCS.

	Empty-body composition	
BCS	**Fat, %**	**Protein, %**
1	3.77	19.42
2	7.54	18.75
3	11.30	18.09
4	15.07	17.42
5	18.84	16.75
6	22.61	16.08
7	26.38	15.42
8	30.15	14.75
9	33.91	14.08

Source: Adapted from [3].

Table 31.3 Shrunk body weight (SBW) change associated with a change in one BCS unit.

Source	SBW/BCS, kg	SBW/BCS, lb
Wagner (1984)	38.0	83.8
Houghton et al. (1992)	33.3	73.4
Buskirk et al. (1992)	37.8	83.4
Graffam (1984)	38.6	85.1
Ferrell and Jenkins (1996)	51.0	112.5
NRC (2000)	44.0	97.0
CSIRO (2007)	46.0	101.4
NASEM (2016)	36.6	80.6
Mean ± SD	40.7 ± 5.82	90 ± 12.8

Source: Adapted from [3].

In situations where access to technology and instrumentation is limited, BCS change can be used as a proxy for weight gain in order to make desired changes in animal body weight. A recent review identified eight published reports in which shrunk body weight change in beef cows was compared with observed BCS change, the latter adjusted to a nine-point scale (Table 31.3) [3, 5, 9, 14–18]. Averaged across the eight publications, change in shrunk body weight per unit of BCS change was 41 ± 5.8 kg (i.e. 90 ± 12.8 lb). In order to hedge risk in underestimating necessary body weight change and also for the sake of simplicity, it is here suggested that this figure be rounded up to 45 kg in shrunk body weight per unit of BCS change (i.e. about 100 lb).

Practical application of these data in conjunction with observed BCS is then straightforward. Consider the following scenario:

1) Moderate BCS (i.e. 5) is desired as a particular beef cow enters late gestation. Late gestation is expected to begin in approximately 90 days.
2) Observation of BCS 90 days prior to expected onset of the final trimester of pregnancy resulted in a BCS of 4 being assigned to the cow in question.
3) Assuming one BCS unit is approximately equal to 45 kg (≈ 100 lb), the cow needs to gain 45 kg (≈ 100 lb) of body weight to reach BCS 5 as she enters late gestation.
4) A daily rate of gain of approximately 0.5 kg (≈ 1.1 lb) is necessary to effect the desired change in BCS (i.e. 45 kg ÷ 90 days or 100 lb ÷ 90 days).
5) A diet is formulated that will allow the cow to gain 0.5 kg daily at a dry matter intake of 2.3–2.7% of body weight.

Most body condition scoring systems for beef cattle were conceived to be visual evaluations of body condition from a distance of up to 30 m from the subject. Published descriptions of each BCS assume that human evaluators have fairly extensive knowledge of bovine anatomy [3]. In addition, extensive training and experience may be required in order to accurately interpret published descriptions of BCS and to assign them correctly to cows and heifers under field conditions.

Even with adequate training, the subjective nature of visual evaluations can lead to significant errors in BCS assignment and in the interpretation of nutritional status of beef cattle [3]. These problems can be exacerbated when several observers are assigning BCS within a single herd. Differences in bone structure between animals, level of gut fill, hide color, hair coat thickness, degree of lighting, and distance between the observer and the animal can contribute to the assignment of an erroneous BCS. Use of a decision-making tree in conjunction with visual appraisal of BCS minimizes the chances that an inappropriate score will be assigned (Table 31.4) [3].

Alternatively, tactile assessment of BCS via palpation removes visual prejudices and, done using appropriate anatomical reference points, minimizes the effects of gut fill on perceived body condition. The authors have developed a simple, rapid palpation system and an associated decision-making tree that remove significant subjectivity from BCS assignment and allow for more consistent scoring between human observers:

1) Animal posture and degree of restraint can influence perceptions of BCS during palpation. Ideally, the animal should be individually restrained, its head should be erect, and handling equipment (e.g. alleys and squeeze chutes) should not be in direct contact with an animal's flanks.

2) Ideally, the same anatomical reference points on all animals should be palpated. Paying close attention to these anatomical landmarks will improve scoring consistency.
3) Locate the withers. With a flat hand, palpate the spinous processes just posterior to the withers.
   a) If spinous processes can be detected with no hand pressure, BCS is 5 or less.
   b) If pressure must be applied to detect the spinous processes, BCS is 5 or greater.
4) Locate the point of the shoulder. Place a hand 30–40 cm (12–16 inches) posterior to the point of the shoulder on the rib cage.
   a) With a flat hand, palpate the ribs at this location.
   b) If ribs are detected with no hand pressure, BCS is 4 or less. Please note that differentiating between thin BCSs (i.e. 1, 2, or 3) requires additional visual assessment of muscling and locomotion (Table 31.4).
   c) If slight pressure allows detection of ribs, BCS is 5 or greater.
   d) Keeping the hand in contact with the same location, tent the fingers and gently move the hide from side to side. If the flesh does not feel spongy or slide easily over the ribs, BCS is 5.
   e) If the flesh feels spongy and slides easily over the ribs, BCS is 6 or greater.
5) If suspected BCS is 6 or greater, locate the tail head. Palpate both sides with finger tips.
   a) If the tail head is flat and firm, BCS is 6.
   b) If there are rippled deposits of fat on both sides of the tail head, BCS is 7 or greater.
6) If suspected BCS is 7 or greater, locate the middle of the rear of the round muscle (i.e. biceps femoris). Run a flat hand down the length of the rear of one round muscle. If a knot-like fat deposit about the size of a small apple is detected approximately midway between the tail head and the hock joint, BCS is 8 or greater.
   a) Verify that both round muscles have these symmetrical fat deposits to differentiate them from scars or inflammation.
7) Watch the cow exit the restraining device. If the animal seems to have significant difficulty moving, BCS is 9.

Advantages of this system include speed, scoring consistency, and relative ease with which it can be taught to others. Conversely, it has two disadvantages compared with visual appraisal of BCS. First, animals must be restrained for body condition to be evaluated via palpation. If coupled with normal processing activities that are associated with weaning, calving, and breeding, this problem is easily overcome. Second, this system is not sensitive to differentiating between BCS 1, 2, and 3. Additional ocular observation is required to differentiate BCS in extremely thin cows (Table 31.4).

Practically, BCS can be noted any time that human handlers are in close proximity to beef cattle; however, the most valuable annual assessment of BCS for breeding females is at or shortly after the point of weaning. The time between weaning and the onset of late gestation presents an opportunity to correct deficiencies in body condition when the number of competing metabolic priorities is at a minimum. In theory, adding to animal body weight during this point of the production cycle should be least expensive and more easily accomplished than at other points of the production cycle that coincide with either late gestation or lactation.

Table 31.4 Decision-making tree for visual assignment of BCS of beef cattle.

	BCS								
Reference point	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Physically weak	Yes	No	No	No	No	No	No	No	No
Muscle atrophy	Obvious	Obvious	Some	Slight	No	No	No	No	No
Fat in brisket	No	No	No	No	Slight	Some	Yes	Yes	Yes
Number of visible ribs	All	All	3 to 5	1 to 2	0 to 2	None	None	None	None
Visible spinous processes	Yes	Yes	Yes	Slight	No	No	No	No	No
Visible hooks and pins	Yes	Yes	Yes	Yes	Yes	Some	Slight	No	No
Fat deposits beside tail head	No	No	No	No	No	Slight	Some	Yes	Yes
Fat in udder	No	No	No	No	No	No	Slight	Yes	Yes
Fat deposits on rear of round muscle	No	No	No	No	No	No	No	No	Yes
Mobility	Lethargic	Slow	Normal	Normal	Normal	Normal	Normal	Slow	Lethargic

Source: Adapted from [3].

Post-Weaning Nutritional Management – Protein and Energy

Given that maintaining a 12-month calving interval is of paramount importance, failure to rebreed in a timely manner is a significant problem on many North American cow-calf operations. Loss of gross income due to delayed rebreeding was estimated to be between $14 and $15 USD for each beef cow in the USA in 2002 [19]; that loss would be expected to be between $20 and $22 USD per beef cow at the time of this writing, assuming a cumulative inflation rate of 42.6% [20].

Timely rebreeding by beef cows is related to pre- and postpartum intake of energy and protein. Prepartum intake of energy and protein may be of greater relative importance than postpartum intake in promoting return to a functional estrous cycle before breeding exposure begins [21]. A likely reason for this observation is that major sources of nutrient demand (i.e. late gestation and lactation) are either minimal or absent during the period between weaning and the onset of late gestation.

Recent research examining performance and reproductive responses to supplementation of forage-based beef-cow and heifer diets with ruminally degradable protein [22–24], ruminally undegradable protein [22, 24–26], fiber-based carbohydrates [27–29], starch-based carbohydrates [27–29], and fats [30, 31] is available; therefore a literature review will not be attempted in this manuscript.

Managers of ruminant livestock should be aware that associative effects between forages and supplements are common, albeit difficult to characterize quantitatively. Associative effects can have strong positive or negative influences on voluntary forage intake, total-tract diet digestibility, and, ultimately, the energy yield of low- to medium-quality forage-based diets [29, 32]. Conceptually, such associative effects represent deviations from the expected additivity in energy yields of feed ingredients combined in a single diet (Figure 31.1). In general, associative effects are not expected to occur when forage quality is relatively high (i.e. CP≥10%) or when the principal energy source in a supplemental feedstuff is based on structural carbohydrate. In these circumstances, the energy contents of forages and supplements are expected to be additive and, thus, predictable.

Associative effects are generally observed when forage quality is relatively poor. When forage quality is poor (i.e. CP<7%), protein supplementation has been associated with larger-than-expected voluntary forage intakes (up to 150% of non-supplemented controls) [33], whereas total digestible dry matter intake may increase by as much as 180% that of non-supplemented controls (Figures 31.1 and 31.2) [29, 34]. Forage intake and digestibility generally increase with

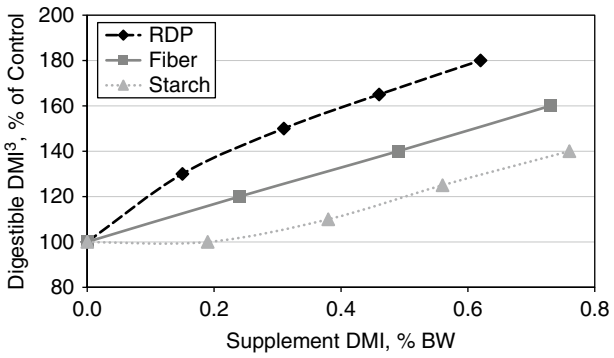


Figure 31.1 Effect of supplement type[1] and intake level on total digestible dry matter intake (DMI) of a medium-quality forage-based diet[2] fed to growing heifers. *Source:* Adapted from [29]. [1] Supplemental ruminally degradable protein (RDP) was delivered at 0, 0.05, 0.10, 0.15, or 0.20% of heifer BW/day using soybean meal (CP = 49.9%, RDP = 65% of CP; DM basis). Supplemental NDF (fiber) was delivered at 0.16, 0.33, 0.49, or 0.65% of heifer BW/day using soybean hulls (66.3% NDF; DM basis). Supplemental ruminally degradable starch (starch) was delivered at 0.10, 0.20, 0.30, or 0.40% of heifer BW/day using dry-rolled corn (total starch = 75%, ruminal starch degradability = 72% of total starch; DM basis). [2] Tall fescue hay (*Lolium arundinaceum*; 8.7% CP, 73.1% NDF, 46.5% ADF; DM basis). [3] Total digestible DMI = DM intake × (% total-tract DM digestibility/100); expressed as a percentage of non-supplemented controls.

protein concentration in supplemental feedstuffs [33–35]. By the same token, high-starch, low-protein supplements have been associated with significant depressions in low-quality forage intake and total digestible dry-matter intake and, therefore, dietary energy yield compared with non-supplemented animals (Figures 31.1 and 31.3) [29, 35, 36]. Development of cost-effective strategies for body condition management in the beef cow requires that managers be aware of these relationships. Beef cows are the most common marketing vehicle for low- to medium-quality forages in North America. Supplemental feedstuffs with modest concentrations of starch and relatively high concentrations of protein should be preferred when beef cows are maintained on low-quality forage diets.

Mineral and Vitamin Nutrition

Poor reproductive performance by beef cows is commonly attributed to subclinical deficiencies of a variety of trace minerals. Although this may be true in certain cases, Corah and co-workers indicated that trace mineral deficiencies were not a common cause of reproductive failure in beef cows [37]. Trace mineral deficiencies appear more likely to occur in high-producing herds and are more likely to affect calf health than dam weight or reproduction [37, 38].

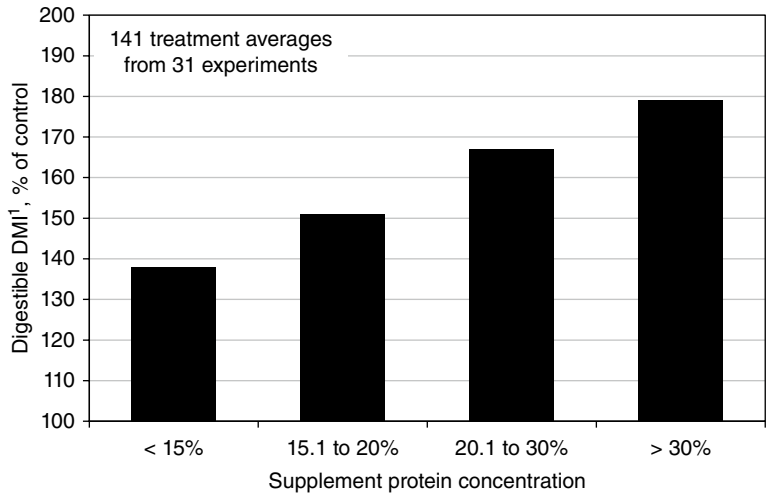


Figure 31.2 Effect of supplement protein concentration on total digestible dry matter intake[1] of low-quality forage (<7% CP)-based diets fed to beef cattle. *Source:* Adapted from [34]. [1] Total digestible DMI = DM intake × (% total-tract DM digestibility/100); expressed as a percentage of non-supplemented controls.

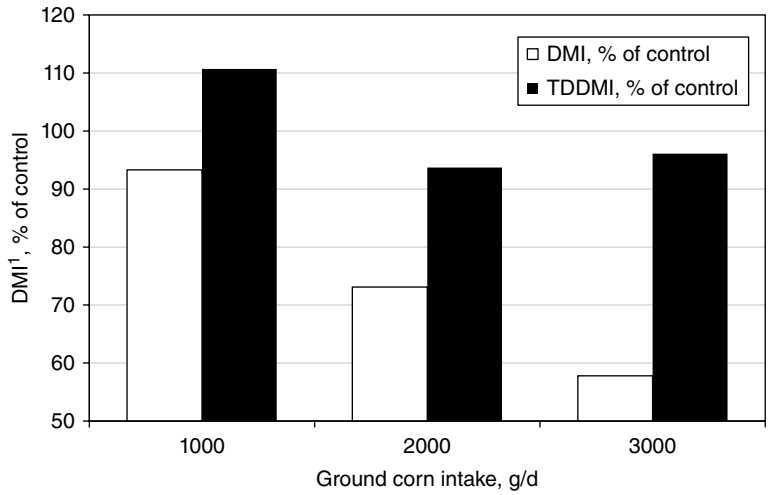


Figure 31.3 Effect of corn grain supplementation on intake[1] of low-quality (4.2% CP) grass hay and on total digestible dry matter intake by beef cows. *Source:* Adapted from [36]. [1] Intake expressed as a percentage of a non-supplemented control of dry matter intake (DMI) and total digestible dry matter intake (TDDMI). TDDMI = DM intake × (% total-tract DM digestibility/100); expressed as a percentage of non-supplemented controls.

There are 25 mineral elements recognized as having significance to beef cattle nutrition [3]. Daily requirements for calcium (Ca), phosphorus (P), sodium (Na), magnesium (Mg), potassium (K), cobalt (Co), copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), iodine (I), and selenium (Se) for beef cattle have been defined. Significantly, only requirements for Ca, P, Mg, K, and Na are definitively changed by gestation or lactation (Table 31.5). Maximum tolerable concentrations have been established for many other mineral elements. Those with potentially toxic or antagonistic physiological effects include chromium, molybdenum, sulfur, aluminum, arsenic, bromide, cadmium, fluorine, lead, mercury, and strontium. Mineral elements most likely to be deficient in beef cow and heifer diets are Ca, P, Na, Mg, Mn, Zn, Co, Cu, I, and Se. In addition, S, Fe, and Mo in water and feedstuffs should be monitored because of their antagonistic interactions with Cu [3, 38].

Many managers of beef cattle supply mineral supplements to match animal appetite without regard for the animal's capability to conserve these elements in organs and tissues. There is clear evidence to support the contention that body mineral reserves can be mobilized in time of need; moreover, deficiencies can take long periods of time to manifest themselves [39]. It is also known that an

Table 31.5 Recommended dietary mineral concentrations and maximum tolerable concentrations (MTC) for beef cows (dry matter basis).

Mineral	Unit	Growing heifers	Gestation	Lactation	MTC
Aluminum	mg/kg	—	—	—	1000
Arsenic	mg/kg	—	—	—	50
Bromide	mg/kg	—	—	—	200
Cadmium	mg/kg	—	—	—	0.5
Calcium[a]	%	0.4	0.26	0.4	—
Chlorine	%	—	—	—	1000
Cobalt	mg/kg	0.15	0.15	0.15	25
Copper	mg/kg	10	10	10	40
Fluorine	mg/kg	—	—	—	40
Iodine	mg/kg	0.5	0.5	0.5	50
Iron	mg/kg	50	50	50	500
Lead	mg/kg	—	—	—	30
Magnesium	%	0.1	0.12	0.20	0.4
Manganese	mg/kg	20	40	40	1000
Mercury	mg/kg	—	—	—	2
Molybdenum	mg/kg	—	—	—	5
Nickel	mg/kg	—	—	—	50
Phosphorus[a]	%	0.2	0.21	0.28	—
Potassium	%	0.6	0.6	0.7	2
Selenium	mg/kg	0.1	0.1	0.1	5
Sodium	%	0.07	0.07	0.1	—
Sulfur	%	0.15	0.15	0.15	0.5
Strontium	mg/kg	—	—	—	2000
Zinc	mg/kg	30	30	30	500

Source: Adapted from [3].
[a] Values are approximate; actual requirement varies with body weight, growth rate, expected calf birth weight, and milk production level.

animal's appetite for minerals is not an indication of its mineral needs [40]. This evidence could be used to argue that mineral supplements should be delivered only to meet a targeted level of daily intake. If overconsumption of free-choice mineral supplement occurs (e.g. a two-week supply of mineral is consumed in one week), additional mineral can be withheld until an appropriate amount of time has elapsed to match the targeted intake level (e.g. two weeks). Similarly, underconsumed free-choice mineral supplements may need to be altered to improve palatability [38]. Overconsumption of mineral supplements can be managed with the provision of additional white salt fed ad libitum. Likewise, removal of white salt or the addition of small amounts of grain or oilseed meals to mineral supplements can stimulate consumption.

Ruminal microbes have the capability to synthesize most water-soluble vitamins (i.e. biotin, choline, B_{12}, folic acid, niacin, pantothenic acid, B_6, riboflavin, and thiamin), which are subsequently available for use in host animal metabolism. In addition, vitamin C (also water soluble) is synthesized *in vivo* by beef cattle in amounts adequate to meet animal requirements; therefore supplementation of ruminant diets with water-soluble vitamins is generally unnecessary [3]. The National Academies of Sciences, Engineering, and Medicine has defined beef-cow requirements for some fat-soluble vitamins (i.e. A, D, and K) but not others (i.e. E). Animals that are exposed to sunlight and fed sun-cured or actively growing forages generally do not require supplemental vitamins A or D. Similarly, vitamin K is synthesized *in vivo* in sufficient quantities to prevent deficiencies and associated problems with blot clotting. Requirements for vitamin E in non-stressed, mature cattle are small and likely met with normal dietary components.

Heifer Development

In modern beef production systems, heifers are expected to first calve at 23–24 months of age. Heifers that calve early in their first calving season tend to rebreed relatively early and wean more and heavier calves during their lives [41]. To accommodate this expectation, heifers should reach puberty by 12–13 months of age to allow one or two complete estrous cycles before breeding at 14 months of age. Age at puberty is influenced by breed, season, and plane of nutrition [41, 42]. Growth rates during the preweaning and postweaning periods are inversely related to age at puberty and postweaning growth rate is proportional to the plane of nutrition [41].

The onset of puberty appears to be associated with increased frequency of pulsatile luteinizing hormone (LH) secretion. Increased energy intake stimulates pulsatile LH secretion, whereas reduced energy intake suppresses LH secretion in heifers [43]. Delayed puberty is also coincident with low concentrations of insulin-like growth factor 1 (IGF-1). Steroidogenic capacities of thecal [44] or granulosa cells [45] are associated with increased concentrations of bovine somatotropin and IGF-1, respectively. Leptin may also act as a regulator of reproductive function and as a mediator for nutritional cues. Short-term fasting of peripubertal heifers decreased leptin gene expression; circulating leptin concentrations were coincident with reductions in circulating concentrations of insulin and IGF-1 and with LH pulse frequency, resulting in delayed puberty [46]. Conversely, serum leptin concentrations were positively correlated with increasing body weight in prepubertal heifers [47]. Addition of ionophores to the diet increases growth rate and tends also to decrease age at puberty, independent of body weight [48, 49].

Once puberty is reached, cessation of cycling requires moderate to severe nutrient restriction. Cassady and co-workers restricted intake of cycling heifers to 30% of NE_m requirements [50]. Heifers that started the restriction period at a BCS of 5 stopped cycling in 61 days, whereas heifers that started restriction at BCS 7 stopped cycling in 148 days. Both groups of heifers lost similar proportions of initial body weight (16–18%).

Historically, it has been recommended that heifers be fed to reach a target of 60–65% of their anticipated mature body weight at first breeding. Heifers fed to a prebreeding target weight of 318 kg (approximately 60% of mature body weight) were younger at puberty; they also had greater pregnancy rates after 20 days of breeding exposure (39 vs 9%) and had greater pregnancy rates at the end of the breeding season (82 vs 66%) compared with heifers fed to a prebreeding target weight of 272 kg (approximately 50% of mature body weight) [51]. Spring-born weaned beef heifers were fed to achieve 55 or 60% of mature weight at breeding. Heifers fed to reach 60% of mature weight were heavier at breeding, they had greater BCS (6.0 vs 5.6; nine-point scale), and more were cycling before the breeding season began (85 vs 74%). In contrast, final pregnancy rates at the end of a 45-day breeding exposure did not differ between groups (88 vs 92%). Heifer performance was then evaluated for three subsequent years; the only measure of productivity that differed between treatments was that 205-day adjusted calf body weights were greater for the heifers fed to achieve 55% of mature weight at first breeding compared with those fed to achieve 60% of mature weight at first breeding [52].

Although there appears to be some elasticity in prebreeding target weights, heifers should be managed to reach 80–85% of mature body weight by first calving [52]. In addition, breeding heifers beginning two to four weeks earlier than mature cows can mitigate reproductive failure at two years of age by allowing more time for uterine involution and resumption of estrus before the second breeding exposure begins.

Bull Nutrition

Rate of gain after weaning and level of nutrition influence the weight and age at which bulls reach puberty. Young bulls maintained on low planes of nutrition had reduced testicular growth, ejaculate volume, sperm production, and seminal vesicle development. Conversely, overfeeding energy to young bulls decreased reproductive performance due to increased fat deposition in the scrotum or the pampiniform plexus. Severe dietary protein restriction also reduced sperm-production capacity in young bulls. Re-alimenting nutritionally stressed growing bulls improved reproductive performance in some trials but not in others. Mature bulls appeared more resistant to dietary stressors than young bulls; however, overfeeding and severe protein restriction resulted in decreased libido [53].

Barth and co-workers conducted a series of experiments evaluating high and low levels of dietary energy prior to weaning or from weaning to puberty [54]. Restriction of nutrients prior to weaning resulted in delayed puberty and reduced testicular size at yearling age; these effects were not observed when plane of energy was altered post weaning. It appears that nutrient intake prior to weaning age has a more profound influence on age of puberty and testicular size than postweaning management.

Minerals influencing the breeding fitness of bulls include Cu, Mn, Se, and Zn. Subclinical deficiencies can negatively affect spermatogenesis [55], sperm motility [56–59], sperm morphology [56–59], testicular development [60], oxidative tissue damage [61], and testosterone biosynthesis [62, 63].

As Cu concentration in semen increased, progressive motility, total antioxidant status, sperm-cell viability following ejaculation, and general fertility of bulls increased [58, 64]. Manganese deficiency has been associated with inhibition of steroid hormone production; severe deficiencies resulted in poor libido, low sperm count, and degeneration of the epididymis and seminiferous tubules [62, 65]. The effects of Mn on reproduction may be related to antioxidant capabilities of Mn-superoxide dismustase [66], which only exists in mitochondria [67]. The activity of this enzyme is depressed when dietary Mn is restricted [68]. Resulting oxidative stress caused decreased mitochondrial membrane potential and poor sperm-cell motility [69].

Adequate Se status is important for normal spermatogenesis, sperm morphology, and sperm motility, independent of vitamin E status [70]. Early work demonstrated a positive association between sperm motility and supplemental Se [71]. More recent research identified a structural selenoprotein (phospholipid hydroperoxide glutathione peroxidase; PHGPx) in the mid-piece that is required for normal motor function of bull spermatozoa. Se deficiency resulted in poor expression of PHGPx, low sperm count, poor motility, and abnormal mid-piece morphology [59, 72].

The Se concentration in bull semen is 16 times greater than that in blood [73]; this may be related to the relatively large mid-piece mitochondrial volume of bovine sperm cells. Bull spermatozoa have greater mid-piece mitochondrial volume than humans, stallions, boars, or rams [74]. Bull spermatozoa also have greater PHGPx activity than other species.

Zinc has multiple roles in male fertility. Zn-deficient diets have been associated with poor sperm-cell plasma membrane integrity, abnormal tail morphology, and impaired motility [57]. Testicular growth of bulls was abnormally low during Zn deficiency but reverted to normal when Zn status was restored [60, 75]. Supplemental Zn also improved morphological and motility characteristics of bull spermatozoa; moreover, adequate Zn status is necessary for testosterone synthesis and normal libido [62, 63, 76].

Gossypol, a compound found in cottonseed-derived feedstuffs, immobilizes bull spermatozoa *in vitro* [49]. Short-term feeding of whole cottonseed or cottonseed meal (i.e. <60 days) is not likely to influence semen quality or spermatogenesis in postpubertal bulls [77]. Conversely, diets high in gossypol may delay puberty and decrease growth in peripubertal bulls [78].

Fetal Programming

Developmental programming is the concept that inadequate or excess maternal nutrition during gestation affects fetal development and has long-term impacts on the offspring [79]. Effects of nutrient restriction during gestation have been evaluated in beef cows more often than nutrient excesses because of the frequency with which pregnant females experience undernourishment in North American beef cattle production systems [3].

Fetal growth and subsequent development are regulated primarily by the placenta, which is developed in early gestation. Restricting beef cows to approximately 75% of metabolizable energy requirements during early pregnancy resulted in lighter fetuses at day 125 of gestation [80]. Additionally, undernutrition during early gestation increased adiposity and suppressed glucose intolerance in offspring [81, 82].

As gestation progresses, energy demand by the fetus increases. The supply of glucose and amino acids to the uterus is essential for proper offspring development [83]. Maternal dietary energy restriction during the third trimester resulted in calves with lighter birth weights and lighter weaning weights than calves from non-restricted cows [84]. Ewes that experienced energy restriction late in pregnancy also birthed lighter lambs [85, 86]. In contrast, growth restriction in lambs did not persist if ewes were adequately nourished during lactation [85].

Although much of the available research on fetal programming has been devoted to understanding how maternal nutrition affects fetal development, maternal nutrition also has an impact on neonatal offspring health and survival. According to the United States Department of Agriculture, 2.3% of calves are born dead and another 3.2% of calves that are born alive die before weaning [87]. There is no evidence directly linking these mortality estimates to developmental programming. Other factors such as weather, predation, and injury contribute to the estimates of calf mortality; however, there is evidence in the literature that prenatal nutrient restriction impacts calf survivability. Corah and co-workers observed increased morbidity and mortality of offspring when dams were restricted to 70% of maintenance energy requirements during the final 90 days of gestation [84]. This can likely be attributed to offspring born at below-average weight having decreased chance of survival in challenging environments [88]. Calves born to thin (BCS 3 and 4; 1–9 scale), two-year-old heifers were less vigorous and had reduced serum immunoglobulin concentrations at 24 hours of age [89]. Maternal undernutrition has been linked to poor colostrum quality and impaired immunoglobulin G (IgG) absorption by calves after birth [89, 90].

Research demonstrates a relationship between prepartum dam nutrition and offspring performance, but the effects are subtle and influenced by the offspring's postpartum plane of nutrition. Mild nutrient restriction is common across cow-calf operations in the USA, yet weaning percentage nationwide is estimated to be quite strong at 83% of females exposed for breeding [91]. Thus, data

describing the impact of developmental programming on current metrics of cow-herd productivity (i.e. pregnancy rates, weaning weights, and weaning percentages) remain equivocal at the time of this writing.

Specific Nutritional Challenges – Pine-Needle-Induced Abortion

In the 1920s, ranchers began speculating that ingestion of needles from the Ponderosa pine tree (*Pinus ponderosa*) were causing cows to abort late-term fetuses. In times of cold weather and increased snowfall, pine-needle consumption by beef cattle was subsequently documented [92]. Since many ranchers in the western USA schedule calving during the late winter and early spring, inclement weather is likely to coincide with the last trimester of gestation. MacDonald conducted the first experiment in which pine-needle consumption by beef cows during late gestation was associated with elevated risk of premature labor [93]. Stuart and co-workers later reported that feeding 0.71–0.85 kg (i.e. 1.5–1.9 lb; DM basis) of pine needles daily to beef cows during late gestation resulted in abortion coincident with retained placenta [94]. Histopathological examination revealed a severe constriction of the caruncular arteries that supply blood to the fetus.

Christenson and co-workers tested the effects of blood plasma collected from cows fed pine needles on placentomes collected from pregnant cows not fed pine needles using an *in vitro* model [95]. Plasma from pine-needle-fed cows increased perfusion pressure in placentomes harvested from cows not fed pine needles, which resulted in increased uterine tone. Increased uterine tone was associated with reduced blood flow to the fetus. Through extensive fractionation, Gardner and co-workers isolated and identified the abortifacient compound in pine needles as isocupressic acid [96]. Isocupressic acid was fed to beef cows during late gestation; a minimum dose of 100 mg/kg body weight twice daily was determined to cause abortion.

Short and co-workers evaluated several dietary factors to determine their effects on voluntary pine-needle intake [97]. Both high-protein and adequate-protein diets were fed to beef cows. The high-protein diet tended to be associated with greater pine needle consumption than the adequate-protein diet. Feeding pine needles that had been left at the site of collection for 12 months, a magnesium supplement, a silage-based diet, pine needles that were aged by grinding and air drying for several months, bentonite, or a complete mineral supplement mixed with pine needles did not change the abortifacient effects of pine needles. Cows evaluated in a 2 × 2 factorial arrangement of treatments (i.e. adequate-protein and high-protein diets, with and without access to salt-sulfur blocks) had an interval to parturition that was intermediate to pine-needle-fed positive controls and negative controls not fed pine needles. Dietary protein concentration or access to salt-sulfur block did not mitigate cow response to pine needles. Gestating cows fed corn silage with ad libitum access to pine needles did not abort, suggesting a possible link between energy intake and susceptibility to pine-needle-induced abortion.

In a later study, Pfister and co-workers evaluated the association between BCS and pine needle consumption [98]. Cows with poor BCS ate more pine needles than cows with adequate BCS. When fed a high-protein, low-energy diet, cows with poor BCS ate consistently more pine needles than cows with adequate BCS. In contrast, poor-BCS cows fed a low-protein, high-energy diet initially consumed more pine needles than cows with adequate BCS; however, differences in pine-needle intake between these treatments decreased over time. Authors speculated that cattle fed elevated levels of protein developed an increased appetite for pine needles because the condensed tannins therein relieved mild sensations of malaise associated with relatively high ruminal ammonia concentrations.

During late gestation, cows grazing in areas where Ponderosa pine trees are abundant are at risk for pine-needle consumption and subsequent abortion. Abortions are a result of decreased uterine blood flow caused by elevated plasma concentrations of isocupressic acid. Relatively high feeding levels of supplemental protein, poor BCS, and inadequate supplemental energy may exacerbate risk of pine-needle consumption and abortion. Therefore cows in the last trimester of gestation should be restricted from access to pine needles. Maintaining adequate energy balance and feeding relatively conservative levels of supplemental protein may also mitigate abortion risk.

Specific Nutritional Challenges – Fescue Toxicosis and Fertility

Widespread planting of tall fescue (*Lolium arundinaceum* [(Schreb.].) Darbysh) across the southeastern USA began in the 1940s [99]. Agronomists promoted establishment of tall fescue because of its persistence across a wide variety of environmental conditions, management, and soil types. Soon after, cattle grazing tall fescue pastures began exhibiting health problems and lesser-than-expected performance; these were later linked to a fungal endophyte (*Epichloë coenophiala*) [100].

The fungal endophyte conveys agronomic benefits to the plant, such as improved grazing tolerance, nitrogen-use efficiency, and water-use efficiency, but it also produces

toxic ergot alkaloids. When consumed by ruminants, ergot alkaloids cause a multifaceted malaise known as tall-fescue toxicosis. Symptoms of fescue toxicosis are generally exacerbated by extremes in ambient temperature [101]. The range of issues associated with fescue toxicosis is beyond the scope of this publication. Readers are directed to the recent review by Roberts and Andrae for a full discussion [102]. This section focuses on the effects of tall fescue toxicosis on beef cattle reproduction and potential strategies to mitigate negative consequences.

Reduced pregnancy and calving rates by beef cows grazing endophyte-infected tall fescue have been widely reported [103]. Reduced forage intake by beef cattle is also a hallmark of fescue toxicosis, which confounds reduced plane of nutrition with reproductive failure in many experiments [104]. Independent of reduced forage intake, ergot alkaloid intoxication increases vasoconstriction and decreases blood flow to limbs and extremities [105]. Poole and co-workers documented ergot alkaloid-induced vasoconstriction of bovine uterine and ovarian blood vessels [106]. Ergot alkaloids also induced a host of hormonal changes in females, which contributed to estrus-cycle irregularities, suppressed hormone secretion, reduced pregnancy rate, and agalactia [107].

One should not overlook the impact of fescue toxicosis on reproductive capabilities of bulls. Looper and co-workers reported that the number of normal sperm cells was reduced by approximately 20% when bulls grazed K31 tall fescue (i.e. endophyte-infected) compared with novel-endophyte tall fescue. Consequently, a greater number of bulls grazing K31 failed breeding soundness examinations [108]. More concerning is decreased scrotal circumference and increased scrotal temperature in bulls exposed to alkaloids. Recently, Page and co-workers reported that spermatozoa incubated with ergot alkaloids (i.e. ergotamine) had reduced cell viability [109]. This suggests a cause-and-effect relationship between grazing endophyte-infected tall fescue and decreased conception rates.

Managing beef cattle successfully in the "Fescue Belt" requires understanding of seasonal ergot alkaloid production so that steps can be taken to reduce alkaloid intake by grazing beef cattle. Bioaccumulation of alkaloids is common and has numerous impacts on cattle health and productivity [110]. Alkaloid production peaks in late spring and is concentrated in seedheads [111]. Plant scientists recommend clipping seedheads before grazing, increasing the biodiversity of pasture plants through interseeding, or conversion of endophyte-infected pastures to alternative varieties that produce few alkaloids or are infected with novel, non-harmful endophytes [112]. Animal nutritionists suggest ameliorating fescue toxicosis by diluting ergot alkaloids in beef-cattle diets through strategic supplementation using non-alkaloid-containing feeds or by timing calving and breeding so as to avoid peak alkaloid concentrations [113, 114].

Other strategies to manage fescue toxicosis have been evaluated. Johnson and co-workers reported that previous exposure to ergot alkaloids influenced stocker cattle performance when fed alkaloid-containing tall fescue [115]. Campbell and co-workers reported a link between a single nucleotide polymorphism for dopamine receptor D2 gene and serum prolactin concentrations in beef cows grazing tall fescue [116]. This suggested the potential to identify cattle with a genetic predisposition for alkaloid sensitivity, although this theory has yet to be validated in the scientific literature.

Many feed additives have been tested for reducing the effects of fescue toxicosis. Much of the data regarding efficacy of feed additives and novel feed ingredients for alleviating fescue toxicosis are proprietary and not reported in the scientific literature. Thus, it is difficult to objectively evaluate their potential role in mitigating fescue toxicosis. Overall, it is unlikely that a single alkaloid avoidance or dilution strategy will resolve symptoms of endophyte-produced alkaloids in beef cattle. A multifaceted approach is necessary to minimize economic losses associated with tall fescue toxicosis.

Summary

Postpartum resumption of estrus and subsequent conception are low priorities, metabolically speaking. They tend not to occur until other priorities such as those associated with maintenance, growth, lactation, and accumulation of basic energy reserves have been satisfied. Nutrient supply prior to parturition and resulting BCS at calving and breeding appear to be the most important factors in determining reproductive performance of beef cows and heifers. Postpartum energy intake above maintenance can improve reproductive performance; however, the least-costly time to build energy reserves in the form of body condition tends to be between weaning and the final 60–80 days of gestation. Early weaning can prevent significant losses in body condition that normally occur during lactation, and has been associated with greater conception to artificial insemination or greater overall pregnancy rates in the subsequent breeding season [117, 118]. Similarly, exposing yearling heifers for breeding two to four weeks before mature cows allows heifers more time to resume estrus before being re-exposed as two year olds.

Nutrient requirements of beef cows vary predictably with the timing of breeding, weaning, and calving. Managers of beef cows and heifers are largely in control of when these

events take place. Sustained reproductive success in the beef cow is predicated on managing the entire production cycle to strategically and economically produce the body reserves of energy and protein needed to support cows through late gestation and peak lactation and to allow resumption of estrus 20–40 days before breeding exposure begins.

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Interaction of Nutrition and Reproduction in the Dairy Cow

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Introduction

Successful reproductive management of the modern dairy cow results from the implementation of best management practices. In the absence of widespread infectious disease, the largest effector of reproductive performance is prior and current nutritional status. Nutritional status of the dairy cow involves a complex interaction between macronutrients and micronutrients and herd-level management. In this chapter we discuss several of these interactions and their effects on reproductive performance. From a veterinary perspective, the primary tenet of animal nutrition is preventative medicine. Cows are programmed to succeed or fail, reproductively, based on the degree of metabolic stress experienced during the transition period from the non-lactating late-gestation stage to the lactating postpartum stage.

Reproductive Goals

The goal of any commercial dairy's reproductive program is the establishment and maintenance of a subsequent pregnancy as soon as physiologically possible following parturition. This pregnancy leads to another parturition and new lactation cycle. Unfortunately, parturition is a biological event associated with considerable risk. The risks associated with several metabolic–immunological disorders are correlated with the transition period of a dairy cow, in addition to the possibility of dystocia which itself has a complex interaction with each of the metabolic disorders. Given the array of possible complications and negative outcomes associated with parturition, profitability is the only impetus strong enough to cause dairy farmers to expose their cattle to this degree of risk. Figure 32.1 displays the relative periods of the lactation cycle with regard to profitability. Over a cow's productive life, the more cumulative time she spends under the area of the curve associated with profitability, the more profitable she is for the operation. The only way to gain access to this phase of the lactation cycle is through additional parturitions.

Transition Period

The transition period is widely accepted as the three weeks immediately before parturition through three weeks immediately following parturition. Disorders associated with this period of the reproductive cycle of the dairy cow include, but are not limited to, periparturient paresis (milk fever), retained fetal membranes (retained placenta), metritis, hepatic lipidosis (fatty liver syndrome), ketosis, and displaced abomasum. These disorders exert both direct and indirect effects on reproductive performance via follicular memory, immune function, and endometrial status. Table 32.1 displays some incidence rates and there follows a discussion of some of these metabolic disorders.

Retained Placenta and Metritis

Placental detachment is an immunologically driven event. In a study by Kimura et al. [1], neutrophils isolated from the blood of cows with retained placenta had significantly lower function before and after parturition, extending up to two weeks after calving. Further evaluation in this experiment revealed lower plasma interleukin (IL)-8 concentrations in cows ultimately developing retained placenta. IL-8 functions as a chemoattractant for neutrophils and induces phagocytosis. Addition of anti-IL-8 antibodies to cotyledon preparations led to a 41% reduction in neutrophil chemotaxis *in vitro*.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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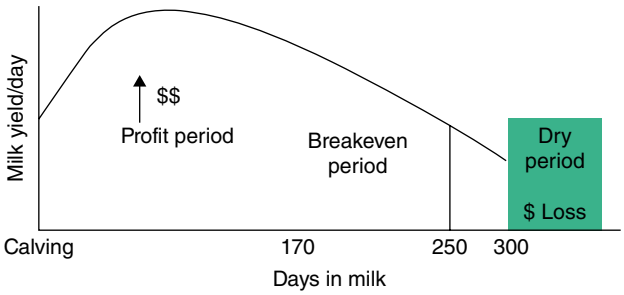


Figure 32.1 Periods of the lactation cycle with regard to profitability.

Table 32.1 Incidence of some metabolic disorders of dairy cows.

Disorder	Incidence (%)
Milk fever	5–10
Retained placenta	5–10
Clinical ketosis	5–10
Metritis	8 to >40
Clinical endometritis (four to six weeks after calving)	15–20

Metritis is the commonly accepted vernacular for an infection contained within the uterus of the postparturient cow. These are typically Gram-negative anerobic bacterial infections caused by invasion (growth) of microbes from the vagina or delivered to the uterus iatrogenically. Immunosuppression during the periparturient period has been shown to predispose the cow to metritis. This immune suppression has been described as starting one to two weeks prior to parturition [2–5]. Cows experiencing dystocia have been identified as being 2.58 and 4.32 times more likely to develop metritis than cows classified as having a normal parturition [6, 7].

One method being studied in an attempt to ameliorate this prepartum immunosuppression is alteration of the ratio of omega-6 ($n-6$) to omega-3 ($n-3$) fatty acids in the duodenum [8]. It appears that by increasing the n 6 : n 3 ratio in multiparous cows before calving, functional properties of mononuclear cells may be improved. It has been suggested that a dietary ratio of n 6: n 3 of 15 : 1 or more could be beneficial to reproductive performance during the lactation to follow.

Ketosis and Hepatic Lipidosis

Negative energy balance (NEB) is defined here as insufficient consumption of the calories necessary to meet metabolic requirements. This is the rule rather than the exception for late prepartum and early postpartum dairy cows. NEB begins in the prepartum period and progresses as dry matter intake (DMI) declines sharply several days before calving. This sharp decline in DMI is now thought to be the result, rather than the cause, of excessive lipolysis [9].

Ketosis is clinically classified as elevated plasma level of the ketone β-hydroxybutyrate (BHB). This occurs when the body's tissues, primarily muscular and neural, utilize less ketone than is being produced by hepatocytes. Increased production of BHB is a result of excessive mobilization of non-esterified fatty acids (NEFA). Ketosis, by itself, is a mammalian survival mechanism. However, animals of economic importance should not be approaching the survival threshold as this is well outside the zone of profitability and efficient production. Cows first testing positive for subclinical ketosis (BHB concentration 1.2–2.9 mmol/l) from three to seven days in milk were 0.7 times as likely to conceive to first service compared with cows first testing positive at eight days in milk or later [10], intimating effect(s) based on temporal occurrence as well as incidence.

Hepatocyte uptake of NEFA depends on the circulating NEFA plasma concentration. Once internalized, NEFA can be utilized, through esterification, and converted back into triglyceride (TG) for internal storage. TG can be repackaged into very low-density lipoproteins (VLDL) and exported from the liver for use by the mammary gland and other tissues. Internalized NEFA can also be oxidized partially, which results in ketone production, or oxidized completely for adenosine triphosphate (ATP) production. This process is termed β-oxidation. Intrahepatic repackaging of TG involves the inclusion of TG into VLDL. The bovine liver has a limited capacity to produce VLDL. Therefore, during periods of excessive lipolysis, the cellular machinery of the bovine hepatocyte becomes overwhelmed with TG and is physically impaired by the amount of TG being stored intracellularly. The upper end of the spectrum of this condition is termed hepatic lipidosis and typically occurs when TG constitutes greater than 5% of liver tissue on a wet basis.

The B vitamins niacin and choline are effectively used to alter the adipose–circulatory–hepatic axis. Niacin effectively limits lipolysis through binding and stimulation of the G-protein-coupled receptor GPR109A in humans [11]. Similar effects have been seen in dairy cattle fed 12 g/day per head of rumen-protected niacin [12]. While effective at reducing lipolysis, this method of control leads to a significant reduction in energy-corrected milk production and is unacceptable in commercial settings. Lipolysis and the subsequent metabolism of NEFA by the bovine liver is an evolutionary adaptation to conserve lactation through provision of VLDL as an alternative energy substrate and provide the substrate necessary for milk fat production in colostrum

and early lactation milk. When this process is limited, production is limited. Although potentially appealing from a reproductive standpoint, ultimately this control strategy is financially infeasible from a commercial perspective.

Supplementation with rumen-protected choline, on the other hand, addresses the rate-limiting step involving the inclusion of TG into VLDL, thus facilitating a net increase in hepatic energy production (translation) without increasing hepatic charge. Hepatic charge has been theorized to be a satiety initiator in the bovine [9]. Choline is integral in the production of phosphatidylcholine and critical for lipid transport and lipoprotein synthesis [13]. Feeding of rumen-protected choline in a study by Lima et al. [14] led to a reduction in mastitis approaching statistical significance. The link between postpartum mastitis and early reproductive performance is discussed later in this chapter in the section "NEFA Concentration and Oocyte Formation." Both choline and the amino acid methionine are methyl donors and can spare each other in this process (Figure 32.2).

Another effective nutritional strategy addressing clinical and subclinical ketosis is the use of the only ionophore currently approved for use in lactating dairy cattle, monensin sodium. In Canada and other countries, controlled-release indwelling capsules that reside in the rumen and provide continuous release of monensin are approved for use. This delivery system is not currently approved in the USA, but feeding of monensin is approved. Monensin has been shown in a meta-analysis to reduce the incidence of clinical ketosis and severity of subclinical ketosis [15]. Monensin exerts its effects by increasing ruminal propionic acid production as a result of bacterial selection. This effect seems counter to the hepatic oxidation theory proposed by Allen [16] as the production of propionate is involved in satiety signaling in ruminants. However, this can be reconciled because the additional production of propionate is spread over a longer timeframe than the spikes from the rapid fermentation of starch following meals [17].

β-Glucans

In its embryonic state in dairy cattle immunology, and infancy in human immunology, immune modulation by β-glucans is addressed crudely from a nutritional standpoint presently. The source and, more importantly, form of the specific β-glucan appears to have significant impact on its functionality regarding gene expression within target immune system cells. Extensive studies are needed to elucidate the exact proinflammatory and proresolving effects of β-glucans and their subsequent effect(s) on reproduction.

Milk Urea Nitrogen

Milk urea nitrogen (MUN) is closely associated with blood urea nitrogen (BUN). BUN is an indirect measure of "excess" dietary protein or, more specifically, ammonia. Within the normal range of commercial ration formulation, BUN concentration increases as the ratio of soluble protein to non-fiber carbohydrates increases. Absolute amounts of the different protein fractions must be matched with their corresponding carbohydrate fractions when considering their respective ruminal degradation rates. When matched, efficient use of dietary protein and carbohydrate results in efficient production of microbial protein with minimal ammonia needing to be detoxified by inclusion into urea by the liver. The biochemical process of converting ammonia to urea exerts an energy cost on the

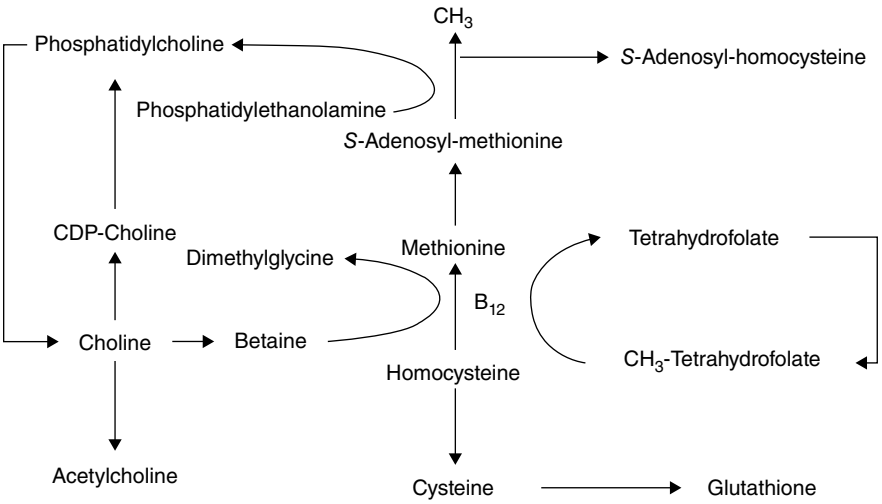


Figure 32.2 Methylation pathways (DNA, proteins, lipids).

cow. A study by Rajala-Schultz et al. [18] showed that cows with MUN levels between 10.0 and 12.7 mg/dl were 1.4 times more likely to be confirmed pregnant than cows with MUN levels above 15.4 mg/dl. In this same study, cows with MUN levels below 10.0 mg/dl were 2.4 times more likely to be confirmed pregnant than cows with MUN levels above 15.4 mg/dl. Studies suggest multifactorial negative fertility effects as a result of elevated BUN, including altered oocyte development [19, 20], embryonic maturation, and intrauterine environment [21, 22].

The use of metabolizable protein and amino acid balancing nutritional models has allowed crude protein and average MUN levels to be reduced. Rumen-protected forms of lysine and methionine are commercially available and appear to be economically viable. Use of these ingredients allows balancing of rations at considerably lower crude protein levels while still maintaining predicted production levels on a metabolizable protein basis since lysine and methionine are the first and second limiting amino acids in normal dairy rations.

NEFA Concentration and Oocyte Formation

The functional micro-unit of reproduction from the female perspective is the ovum. Follicular memory is a carry-over effect that delays the influence of metabolic status on reproductive performance by as much as 60 days [23–25]. As the investigation and understanding of the influence that individual fatty acids exert on reproductive performance increases, NEB becomes a more complex proposition as it relates to fertility in the dairy cow. The negative effects of oleic acid, and a combination of oleic, palmitic, and stearic fatty acids, have been studied with regard to their direct effect(s) on granulosa cells and cumulus–oocyte complexes [26] and theca cells [27]. *in vivo*, these negatively correlated effects are secondary to elevated circulating plasma levels of these individual and/or combinations of NEFA. Fatty acid composition of adipose tissue is influenced greatly by diet and degree of ruminal biohydrogenation of unsaturated fatty acids. Since NEFA result from the liberation of TG from adipose tissue, nutritional composition of absorbed fatty acids has a direct influence on the relative and absolute concentrations of NEFA.

Setting aside the direct influence that individual circulating fatty acids may exert on the ultimate fertility of the ovum, elevated circulating NEFA may be linked to runaway inflammatory response and insulin resistance, the latter being part of a vicious positive feedback loop that results in an ever-increasing NEFA concentration. Feeding of the omega-3 fatty acids α-linolenic acid and docosahexaenoic acid may directly dampen this hyperinflammatory state as a result of binding proliferator-activated receptors [28]. Studies completed recently have shown a decrease in embryonic loss and concomitant increase in milk production in cows supplemented with the essential fatty acids eicosapentaenoic acid and docosahexaenoic acid. Current reasoning for these results is a significant decrease in the caloric requirements of the cellular immune system, due to modulation, in these postpartum cows so that glucose is non-selectively spared, as glucose is the primary energy source for macrophages and neutrophils. As the bovine liver has a finite capacity for glucose production, primarily via gluconeogenesis, this sparing effect should return the postpartum cow to a positive energy balance at lower days in milk. In the bovine, resolution of NEB leads to reestablishment of normal ovarian cyclicity. On a molecular level, an intracellular shift from the production of proinflammatory eicosanoids to more pro-resolving eicosanoids likely explains the responses seen in these studies. This shift would break the positive feedback loop of proinflammatory eicosanoids leading to radical oxygen species production, tissue destruction, and further proinflammatory eicosanoids.

As alluded to above, tumor necrosis factor (TNF)-α production by adipocytes, and macrophages residing within adipose tissue, could enhance insulin resistance. Studies using exogenous TNF-α in cattle have led researchers to this conclusion [29–31]. This is especially significant in a transition cow that already has lower circulating insulin levels and decreased insulin sensitivity compared to that of a normal mid-lactation animal. One can see the possibility for a positive feedback loop here as well. It is possible this model explains obesity-induced diabetes in humans and the progression from normal periparturient lipolysis to a hyperketotic/hepatic lipidosis state in dairy cattle.

An indirect association between ketosis and reproductive efficiency, via clinical mastitis, can be created by a systemic inflammatory state. Cows experiencing ketosis have been shown to have twice the risk of developing clinical mastitis [32] and cows that experienced a case of clinical mastitis within 30 days after insemination were 16% less likely to conceive on first-service insemination. Cows experiencing subclinical or clinical mastitis prior to first-service insemination show increased days to first service, days open, and reduced first-service conception rate [33]. It is not difficult to visualize an impaired corpus luteum, hampered by proinflammatory prostaglandins, unable to produce progesterone at a level sufficient to maintain an early pregnancy.

Nutritional Heat Stress Abatement

Heat stress, a result of the cow's physiological response to elevated temperature humidity index (THI), has been shown to lower conception rates [34] and pregnancy rates [35]. Lactating dairy cattle typically begin to experience heat stress around a THI of 70. This stress is exacerbated by dietary fiber digestion. Per unit of dry matter, the ruminal degradation of neutral detergent fiber (NDF) carries the highest heat increment of any of the macronutrients found in dairy rations. Ether extract (EE) carries the lowest heat increment of digestion. Therefore it is common practice to adjust lactating rations to reduce NDF levels and increase EE levels in response to times of intense heat stress. This serves two purposes: it increases the caloric density of the ration at a time when the cow is expending additional calories in an attempt to maintain core body temperature, and it decreases the heat increment that would have been created by digestion of the marginal fiber. Care must always be taken to avoid ration extremes that would result in fiber levels that are too low and/or EE levels that are too high; these extremes would put cows at risk of ruminal acidosis and decreased fiber digestibility/depressed DMI, respectively.

An additional method of nutritionally cooling cows has been studied [36] in beef cattle and could have application in a dairy setting. It involves feeding an osmotically active ingredient during times of heat stress and has been shown in at least one controlled study to minimize the degree of body temperature elevation. More work is needed on this concept to determine its efficacy and feasibility.

Body Condition Score

Probably the most complex phenotypic expression, body condition score (BCS) of the dairy cow is a combination of genetic predisposition for both reproduction and production, and numerous variables associated with management decisions. Elevated BCS is usually associated with either prolonged days to conception or abortion, extending length of lactation in both cases. This allows these cows to remain on diets that are higher in energy density than needed for maintenance due to decreasing milk production, as the lactation curve progresses at approximately a negative 9% monthly slope. Cows that are obese (BCS > 3.75 on the five-point scale) at calving tend to have higher risk of metabolic disorder [37], extending the period of NEB and delaying conception. Current recommendations are to have cows at a BCS of 3.0–3.25 at the time of parturition. From a univariate standpoint, this management strategy does decrease the incidence of certain energy-related metabolic disorders. However, reducing the available energy reserves for the ensuing lactation could have negative effects on the lactation curve. Other strategies, such as choline supplementation in the postpartum period, could prove to be a more profitable pathway in the management of NEB. This axis between nutrition, reproduction, and body condition can be either coupled and provide a basis for continuous improvement in efficiency or decoupled, leading to challenges and economic loss.

Methionine

As an essential amino acid, and initiating amino acid in virtually all mammalian proteins, methionine supplementation is common in commercial dairy cattle. Sufficient data have accumulated since the previous publishing of this text to justify a discussion of methionine and its effects on the reproductive performance of the female bovine. Numerous pathways elucidated by researchers have shown increased embryonic retention in response to methionine supplementation. Toledo et al. in 2015 [38] compared multiparous cattle that were supplemented (treatment) or not supplemented (control) with 21.2 g/day of Smartamine® M from ~30 to ~126 days in milk (DIM). Cows supplemented with methionine during that period experienced a 6.1% pregnancy loss between pregnancy days 28 and 61 compared to 19.6% pregnancy loss compared to their non-supplemented counterparts at a p-value of 0.03. Embryonic abdominal diameter (5.9 mm vs 5.3 mm) and embryonic volume (209.3 mm^3 vs 160.5 mm^3) were both positively affected, with p-values at 0.008 and 0.005, respectively, on day 33 of pregnancy. In this study there were no significant differences measured in the reproductive performance of primiparous cows. Acosta et al. in 2016 [39] showed a higher cytoplasmic lipid concentration (7.02 ± 1.03 arbitrary units (AU) vs 3.61 ± AU) in embryos flushed 6.5 days after artificial insemination in Holstein cows supplemented with Smartamine M at 0.08% of the ration dry matter from 21 days prepartum to 30 days postpartum. The researchers proposed that this higher embryonic lipid content in the preimplantation period would be an explanation for the increased embryonic survival in methionine-supplemented animals.

Baez et al. have shown larger uterine size and decreased fertility in multiparous cows when compare to primiparous cows [40]. Baez et al. also have shown a decreased tendency in reproductive performance in recipient heifers with a larger uterine size [41]. It is rare that a single phenotypic expression can explain the response to an individual nutrient. But it appears in the case of methionine, and perhaps other essential amino acids, that the variable uterine

volume of the dam hosting the conceptus could determine the absolute dietary requirement for this amino acid. Increased uterine fluid concentration of essential and nonessential amino acids in the preimplantation phase of pregnancy was shown by Groebner et al. [42]. Genomic identification of this trait could influence the efficiency with which methionine is utilized for manipulation of the bovine pregnancy.

Minerals and Vitamins

Proper nutrition and management during the transition period are crucial for the prevention of postpartum reproductive disorders. The role of proper energy and protein supplementation in the prevention of uterine disorders is well defined, but mineral and vitamin balance and supplementation should not be ignored [1].

Cows suffering from clinical or subclinical periparturient hypocalcemia are more at risk of experiencing secondary disorders such as dystocia, uterine prolapse, retained placenta, and metritis [43–46]. The pathogenesis and prevention of parturient hypocalcemia or milk fever continues to be a topic of debate and research. However, current management practices to prevent parturient hypocalcemia attempt to reduce the physiological lag time between the potentially hypocalcemic demands of lactation and recovery back to a functional normocalcemic state.

Factors contributing to hypocalcemia include excessive prepartum dietary levels of calcium (1.35% DM), excessive levels of dietary phosphorus, low dietary levels of magnesium, increasing age, and breed – with Jersey cattle being at increased risk [43, 47]. The most commonly recognized presentation of milk fever (clinical hypocalcemia, parturient hypocalcemia) is during stage 2 and is diagnosed in the bovine by the clinical signs of recumbency, cold extremities, and a weak rapid pulse. Total serum calcium less than 8.0 mg/dl is considered diagnostic of hypocalcemia in the bovine [48]. The traditional nutritional method of limiting the incidence of clinical and subclinical hypocalcemia involved limiting calcium intake before calving so that the total absorbed calcium per head was less than 20 g/day. This stimulated bovine parathyroid hormone (PTH) secretion and increased bone resorption of calcium. This method has largely been replaced with the advent of dietary cation–anion difference (DCAD) diets. The equation for DCAD is (Na + K) – (S + Cl). Since sodium intake is held at a minimum during the immediate prepartum period as a means to limit udder edema and feeding excessive sulfur has negative implications, this equation can, practically, be simplified to (K – Cl). Anionic salts such as ammonium chloride, calcium chloride, and magnesium sulfate (used primarily as a magnesium source but also as an acidifier) are used to create a state of compensated metabolic acidosis. Newer commercial ingredients, usually a protein carrier treated with either hydrochloric acid or an organic acid, or a combination of organic acids serve the same function as the anionic salts. This method of hypocalcemia prevention has been described by Zeisel and Holmes-McNary [13]. Excessive acidification is undesirable as it increases the cow's caloric requirements in an effort to compensate for the greater degree of metabolic acidosis. This usually comes in the form of increased respiratory rate in an effort to reduce acid load. Decreased DMI is also associated with compensated metabolic acidosis and worsens with the degree of acidosis. Cows are typically fed these acidifying diets during the three weeks immediately before calving, when they are already mobilizing adipose tissue and declining in DMI. Diligent monitoring of urine pH as a measure of the degree of compensated metabolic acidosis is necessary in order for this program to be successful.

Since last publication, a novel product has been introduced to the market for the control of hypocalcemia. A synthetic zeolite, X-Zelit®, has been added to the list of feed ingredients available. Naturally occurring zeolite has been used for years as a mycotoxin binder and in the amelioration of ruminal ammonia effluence. Its porous 3-D structure lends itself to the binding of a plethora of molecules varying greatly in molecular mass. Fed to the prepartum bovine, this product binds calcium, phosphorus, and magnesium. In the author Cargile's experience this multifaceted approach is more effective in preventing peripartum hypocalcemia and hypophosphatemia than induced preparturient compensated metabolic acidosis. Care must be taken when using this product to provide additional magnesium supplementation and to provide micronutrients in a protected form to prevent iatrogenic hypomagnesemia and/or temporary micromineral deficiencies. The author feeds between 0.45 and 0.50% magnesium on a dry matter basis when utilizing this product.

Magnesium plays an essential role in calcium homeostasis and as a result may contribute to hypocalcemia when dietary levels are insufficient [47, 49]. Hypomagnesemia can blunt PTH secretion in response to low calcium and interfere with the interaction of PTH and its target tissues [49]. High dietary potassium will reduce the absorption of magnesium in the rumen as well as reduce the effectiveness of an anionic salt program. It is recommended that the magnesium content of the close-up ration should be between 0.35 and 0.4% of dry matter to ensure adequate absorption [49].

Trace minerals are present in the tissues at very low concentrations and their requirements are expressed in milligram or microgram amounts. Trace minerals considered to be of importance in dairy cattle include cobalt, copper, iodine, iron, manganese, molybdenum, selenium, zinc,

and possibly chromium and fluorine [43]. Trace minerals are components of metalloenzymes, hormones, and cofactors of functional proteins, or enzymes that are involved in many aspects of energy and protein metabolism, hormone synthesis, and immune function [43, 50]. Infertility in cattle, including decreased ovarian activity, reduced conception rates, increase in dystocia, abortions, and delayed onset of puberty, is linked to specific enzymatic dysfunction resulting from one or more trace mineral deficiencies [51]. In the bull, trace mineral deficiency is associated with a decrease in spermatogenesis, decrease in libido, increase in abnormal sperm, and a reduction in testicular size [44, 50]. Trace minerals affect reproduction directly or indirectly through multiple functional proteins and enzyme systems. For example, selenium (Se) is well known for its role as a cofactor in the enzyme glutathione peroxidase, an important antioxidant that is present throughout bodily fluids and organs, and within cells including sperm and cells of the ovary [50]. Such antioxidant activity helps maintain cellular function in the face of oxidative stress, improves cellular immunity, and, when supplemented with other antioxidants such as vitamin E, can improve fertilization, reduce retained fetal membranes, and reduce the incidence of cystic ovarian disease [52, 53].

Vitamins like trace minerals are cofactors. Vitamins required by dairy cattle are classified as being fat-soluble (A, D, E, and K) or water-soluble (B vitamins and vitamin C) [43]. Water-soluble vitamins are produced by rumen microbes and true deficiencies in cattle with functioning rumens are rare [43]. Vitamins A and E are the only ones with a true dietary requirement because vitamin D is produced through ultraviolet (UV) radiation of the skin and vitamin K by bacteria of the rumen and intestines [43]. However, a reasonable practice includes supplementation of dry cows with 20 000–30 000 IU vitamin D daily to ensure proper calcium absorption [49] and compensate for animals not receiving sufficient UV radiation for de novo vitamin D synthesis. These conditions are common in dairy cattle residing in extreme northern and southern latitudes. Vitamins and trace minerals also share a synergistic relationship. Vitamin E and selenium have a sparing action on the requirement of the other and share the same characteristics [54]. Both contribute to antioxidant activity and maintain cellular health and function. Selenium contributes to intercellular, intracellular, and extracellular activity, while vitamin E preserves cellular integrity by protecting the cellular membrane [50].

Trace Mineral and Vitamin Supplementation Challenges

As discussed, trace minerals and vitamins are essential for proper immune function, cellular health, and hormone synthesis, which are essential for postpartum involution, health of the reproductive tract, and resumption of normal estrous cyclicity after calving. It has been established previously that fresh cows suffering from postpartum diseases such as retained fetal membranes and metritis experience poor reproductive performance and increased days in milk [6]. However, compounding this issue is that approximately one month prior to calving, there is maternal transfer of stored trace minerals from the dam to the fetal tissue. Studies have demonstrated this transfer with copper, manganese, zinc, and selenium, which are the most limiting trace minerals with regard to fetal and neonatal survival and development [55–57]. In addition, transfer of vitamins A and E in colostrum occurs at calving and colostrum is a source of these vitamins for the calf after birth [57, 58]. Maternal transfer of trace minerals is essential for calf health, but this transfer occurs at the cow's expense during a time of decreased DMI and demands on the immune system that occur prior to calving [59]. Further, contributing to the risk of deficiency and disease is the issue of mineral antagonism or negative mineral interactions, which reduces absorption in the gastrointestinal tract as well as antagonistic activity systemically. Excessive sulfur found in dried distillers grains, molasses, and water reduce copper absorption in the gastrointestinal tract through formation of copper sulfide [60, 61]. Selenium and sulfur are physically and chemically similar and when excessive sulfur is present in the ration, competition for absorption can result in reduction of selenium absorption [62]. In addition to sulfur, calcium levels greater than 1% of ration dry matter reduce the absorption of selenium from natural sources [63]. Systemic antagonism of the enzymatic function of copper can occur if excessive consumption of sulfur is combined with excessive consumption of molybdenum, resulting in the formation of thiomolybdate in the rumen that reduces copper absorption in the gastrointestinal tract [64, 65]. In addition, thiomolybdate is absorbed and blocks systemic function of copper and increases biliary excretion of copper from liver stores, resulting in symptoms of copper deficiency [60].

Subclinical deficiencies of trace minerals that occur due to antagonism, maternal transfer, or reduction in DMI prior to calving can persist into lactation, potentially compromising reproductive performance. Nutritionists address the risk of trace mineral deficiencies by providing proper amounts of trace minerals in the ration and reducing potential negative mineral interactions or antagonism, as well as including trace minerals with improved bioavailability such as chelates or organic trace minerals [60]. The study of trace mineral form, function, and supplementation continues to be an active area of research. Recent studies have shown a benefit to health when trace minerals are

provided as an additional supplement or parenterally administered to cows that are provided with a supplemented ration. Such benefits to a cow's reproductive and physical health have been demonstrated with supplementary selenium and vitamin E. In addition, recent studies have shown an improvement in uterine health and reduction in the population of bacteria causing metritis when an injectable trace mineral product containing zinc, manganese, selenium, and copper was administered during the dry period and during early lactation [52, 53, 66, 67].

Summary

Through genetic selection, economic pressure for higher milk production, and maintenance of profitability, today's dairy cow has become a physiologic athlete. However, development of today's dairy cow has resulted in many challenges to reproduction and health. Continually addressing those challenges through research has allowed better understanding of how to preemptively design rations and herd health protocols to allow for a successful lifetime.

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Cystic Ovarian Follicles

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Introduction

Cystic ovarian follicles (COF) have been recognized as a condition that compromises normal ovarian cyclicity and is a significant disrupter of normal reproduction in cattle. Frequently a cause of subfertility and reduced reproductive performance, it has been recognized in cattle for over 100 years. This condition is most commonly characterized by anovulation and is noted to occur in a number of mammalian species. The condition in cattle results in a major cause of economic loss worldwide, most notably in dairy cattle. Cows in modern dairy production systems are under intense metabolic and environmental stresses that can lead to compromised reproduction, with COF being one of the most common manifestations encountered. The major causes of economic loss attributable to this condition involve: increased days open in the postpartum period leading to extended calving intervals; increased drug and treatment costs associated with its management; increased semen costs attributable to the increase in servicers per conception; and higher culling rates of affected animals [1, 2]. One study in dairy cattle demonstrated that cows with COF took 6–11 days longer to reach first service and took 20–30 more days to conception than control cows, clearly demonstrating the economic impact of this condition [3].

Terminology

Numerous terms and definitions have been used to describe the anovulatory condition of cattle, which is most commonly termed cystic ovarian disease (COD). Other terms that have been used previously to describe the same clinical manifestation include adrenal virilism, nymphomania, ovarian cysts, cystic ovarian degeneration, and cystic ovaries, among others [2]. The classic definition proposed by Roberts [4] was the presence of an anovulatory structure on the ovary more than 2.5 cm in diameter and which has persisted for at least 10 days in the absence of a corpus luteum (CL). Several aspects of the former definitions are relatively arbitrary and do not reflect our current understanding of the condition. Cattle typically ovulate a dominant follicle with an average follicular diameter of 1.6–1.9 cm. The use of a size limit of 2.5 cm thereby excludes all cystic follicles which are smaller than this arbitrary size [5]. Also, many previous definitions stated the requirement that the cystic structure be present for a minimum of 10 days. This requirement should also be questioned as it is known that these structures are dynamic in nature. Evidence shows that both naturally occurring as well as steroid-induced cystic follicles undergo frequent turnover and are replaced by a new and similar structure at another site on the same or opposite ovary. Current management systems would often not allow the cow to be reexamined to fulfill the 10-day duration requirement. Older definitions have also required the absence of a CL. The necessity for the absence of a CL is not universally fulfilled as it is evident that some cysts are not steroidogenic and thus are hormonally inactive and would not necessarily influence the estrous cycle. Such cystic structures can be found in the presence of a functional CL.

Vanholder et al. [5] attempted to more accurately define and describe this anovulatory condition of cattle and its effects on reproduction as "cystic ovarian follicles." COF is defined as the presence of a follicle(s) with a diameter of at least 20 mm, present on one or both ovaries in the absence of any active luteal tissue, and which clearly interferes with normal ovarian cyclicity [5].

Further subclassification of the COF condition into follicular or luteal cyst is based on the degree of luteinization and on progesterone levels in blood or milk. Follicular

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

cysts are typically thin-walled structures that secrete varying amounts of estradiol. The thickness of the layers of granulosa cells dictates the amount of estradiol present in the intrafollicular fluid. Both cyst types are considered to be different forms of the same condition, with luteal cysts being a follicular cyst in which theca and granulosa cells have undergone some luteinization and are producing progesterone [2]. Follicular cysts do not secrete progesterone, whereas luteal cysts secrete varying amounts of progesterone depending on the degree of luteinization. A single universal threshold level of progesterone used to distinguish follicular cysts from luteal cysts has not been established [5]. The ability to accurately classify each individual cyst as a follicular or luteal cyst remains subject to personal interpretation of the clinical examination and history findings in addition to ovarian ultrasound findings and serum progesterone levels.

Incidence

The incidence of COF in cattle is very dependent on their intended use and their respective management system. The incidence of COF is much greater in dairy cows compared with beef cattle and dairy heifers, where the incidence is relatively low. Utilizing data from a number of studies, the average incidence of COF in lactating dairy cattle is estimated to be near 10–12%, with various studies reporting incidences ranging from 3 to 32% [2]. There are numerous factors that can impact the apparent incidence of COF. The time of diagnosis can easily influence the apparent incidence of COF as it has been shown that as many as 60% of dairy cattle that develop COF recover spontaneously prior to their first postpartum ovulation and may never be diagnosed. Cattle that experience a disruption in normal parturition, fetal membrane release, or any metabolic disease in the postpartum period are at almost two times greater risk of developing COF than normal control cattle.

In beef cattle the incidence of COF is much lower when the cattle are under routine management. It is when the reproduction of beef cattle is intensively managed or manipulated that the incidence of COF begins to rise above that of the general population. Beef cattle used for embryo donation after superovulation appear to develop COF at a much higher incidence than the general population and may approach the incidence rate of dairy cattle.

Pathophysiology of COF Formation

Potential mechanism(s) involved in the pathogenesis of COF have been investigated for over 50 years, but to date many studies have not yet yielded the single definitive cause resulting in the condition of COF. A general consensus is that there is an underlying likely multifactorial dysfunction or neuroendocrine imbalance involving the control mechanisms of the hypothalamic–pituitary–gonadal axis resulting in ovulation failure [1, 2, 6–13]. The precise or primary mechanism(s) leading to ovulation failure remains elusive. A complicating factor in this search is that it is evident that there are multiple factors/pathways/imbalances involved in the pathogenesis of this condition. These factors can be lumped into one of the following: individual genetic factors, phenotypic traits, environmental factors, metabolic and cellular factors, and management factors, which can individually and/or collectively be involved in contributing to this condition [5].

The most widely accepted hypothesis involves the altered release of luteinizing hormone (LH) from the pituitary gland. The preovulatory surge of LH is absent, insufficient in magnitude, or improperly timed, leading to failure of the dominant follicle to ovulate. The dominant follicle continues to grow and becomes large and anovulatory [1, 2, 5, 14]. There does not appear to be a reduction in gonadotropin releasing hormone (GnRH) content in the hypothalamus or a reduction in GnRH receptors in the pituitary [5]. LH content in the pituitary also does not appear to be reduced in cows with COF [5, 15]. Injections of exogenous estradiol to cows with naturally occurring or induced follicular cysts fail to induce an LH surge, although an injection of GnRH in affected cows can induce the release of LH [16–19]. These findings support the belief that there is a functional breakdown in the positive estradiol feedback loop which controls LH secretion. There appears to be disruption in the responsiveness of the hypothalamus to estradiol which leads to resultant ovulation failure. Under normal conditions, preovulatory follicles secrete estrogen that has a positive feedback effect on the hypothalamic–pituitary–ovarian axis, inducing an LH surge which is responsible for ovulation of the dominant follicle [16]. Because of the functional abnormality in the regulation of LH secretion, cows with COF lack an adequate LH surge needed for ovulation, which results in ovulation failure.

In cows with COF, there is also loss of the negative feedback effects of progesterone, leading to relatively high levels of LH pulse secretion. These LH pulses have been observed to be higher than those in cattle with a normal luteal phase. High LH pulses promote continued and excessive growth of the dominant follicle, ultimately leading to the abnormally large preovulatory follicle [16, 20, 21]. Additionally, the period of estradiol and inhibin production by the dominant follicle is protracted, which helps establish long-term dominance of the cystic follicle via suppression of follicle-stimulating hormone (FSH) production by the anterior pituitary [16, 22–25]. When the COF ultimately undergoes regression and no longer produces sufficient estradiol and

inhibin at levels sufficient to suppress FSH, a new follicular wave emerges. Unless the abnormality in the estradiol feedback loop is corrected, the new dominant follicle of the subsequent wave will also become cystic and anovulatory, thereby sustaining or perpetuating the anovulatory condition [16]. This apparent turnover and replacement of the COF condition can be responsible for the protracted anovulation noted in some cows.

There also appears to be an intraovarian component in the pathogenesis of COF. Metabolic hormones, most notably insulin and insulin-like growth factor (IGF)-1, stimulate follicular cell steroidogenesis and proliferation. Growth of dominant follicles is dependent upon IGF-1 helping drive estradiol-17β secretion. Insulin is needed in the promotion of follicular maturation [26]. It is known that cows with COF are often concurrently or were previously exposed to various kinds of insults or stress such as oxidative stress, negative energy balance, dietary restriction, reduced or poor liver function, and low circulating insulin and IGF-1 [26–29].

There has been an association with these stressors and an increase in heat shock protein (HSP) in the ovaries of cows with COF. It is speculated that the altered expression of HSP genes decreases apoptosis in the follicular wall and leads to the delayed regression of cystic follicles [27]. Although the precise relationship between the aberrant amounts of HSP in the ovaries of cattle under stress and the COF condition is not completely understood, it is speculated that there is an association with HSP and an intraovarian component of COF pathophysiology [27].

Several studies have looked at the impact of non-esterified fatty acids (NEFA) on the formation of the COF condition. Often during early lactation when dietary intake is insufficient to meet the need for milk production, NEFA levels are high. High NEFA has been proposed as a contributor in COF due to its effects on follicular growth and development related to its cytotoxic effects on bovine theca cells and their steroidogenesis. The presence of elevated NEFA and the sustained effects on the follicular cells is proposed to be a potential explanation for cases that fail to recover spontaneously and cows that repeatedly develop the condition [30].

Clinical Signs

The clinical sings associated with COF are generally related to altered reproductive behavior and, less frequently, changes in physical characteristics. Most commonly, cows with COF show prolonged periods of anestrus, which is seen in over 80% of affected cows. This is most evident in cattle that develop COF early in the postpartum period [4]. Nymphomania with prolonged periods of persistent and frequent estrus is seen in approximately 10% of cows with COF. It is common for the affected cow to frequently participate in riding activity of estral cows but not stand for mating herself [4]. The presence of an extremely masculine phenotype has also been associated with cows with COF who show prolonged nymphomania [31]. It appears that as the interval from calving to diagnosis of COF increases, the likelihood of observing nymphomania behavior increases [4]. Estrous cycles with sporadic and irregular patterns can also be the primary clinical sign in cattle with COF.

Diagnosis

Transrectal palpation of the reproductive tract has been the primary means of diagnosing COF for many years. The accuracy with which even an experienced clinician can correctly determine the specific type of cyst present is relatively poor. Transrectal ultrasound can be used to more accurately determine the specific type of cyst present. Follicular cysts typically have a thin wall (≤3 mm), whereas luteal cysts typically have a thicker and more echogenic wall (≥3 mm). The follicular fluid is often hypoechoic in follicular cysts, whereas with luteal cysts echogenic strands may be present, creating a cobweb-like appearance [32]. The collective findings of transrectal examination of the reproductive tract including ultrasonography, blood progesterone levels, and the clinical history of the cow will allow the most accurate diagnosis regarding the type of cyst present. It is relatively uncommon for blood progesterone levels to be determined in the diagnosis and management of routine cases in clinical practice.

The accuracy with which a skilled clinician can identify the type of cysts present based on palpation alone is relatively poor [33]. The dynamic nature of both cysts and developing corpora lutea can complicate the diagnosis when palpation alone is used. Farin et al. [34] showed that 10% of cows diagnosed as having cysts based on transrectal examination were found to have a structure consistent with a normal CL when the ovaries were subsequently examined with ultrasound. When ultrasound technology was used the accuracy of a correct diagnosis of cyst type was 74% for follicular cysts and almost 90% for luteal cysts [35]. Progesterone concentrations have been shown to correlate very well with cyst wall thickness, with 3 mm often being the threshold between follicular and luteal cysts [1, 36]. When blood progesterone concentration is combined with both palpation and ultrasound findings, the diagnosis of cyst type approaches 100%. Knowledge of progesterone concentrations could certainly aid in treatment decisions, although most of the currently recommended treatments effectively treat both luteal and follicular cysts, thereby negating the need for knowing this information prior to treatment.

Treatment Strategies

Many techniques, drugs, and strategies have been used in the treatment of COF in cattle over the last hundred years or so. Currently the most effective and most widely used treatments all involve hormonal therapy(s) of some kind which are aimed at causing ovulation of a dominant follicle to occur or to reestablish the responsiveness of the hypothalamus to the positive feedback of estrogen. Older therapies that are no longer recommended include ovariectomy, uterine infusions of antibiotics or antiseptics, and crushing of the COF. Studies in the 1950s showed that when compared with hormonal therapies of the time, manual crushing showed no advantage and there may even be some adverse conditions such as trauma and adhesions associated with its use [37]. Fatal hemorrhage associated with attempts to crush or manually rupture COFs has been reported [38]. This form of treatment, although still found in several texts, should never be used as a primary method of treatment due to the potential side effects as well the availability of safer and more reliable and effective treatments.

GnRH

Hormone therapy aimed at either causing (GnRH) or mimicking (human chorionic gonadotropin) an LH surge can be used to treat follicular cysts. Of these two options, GnRH is generally chosen first due to its low molecular weight and size, which reduces the likelihood of an adverse immune reaction [1, 7]. The standard dose of 100 μg was shown in a pharmacokinetic study to be more than adequate for the production of an LH surge capable of triggering ovulation of a dominant follicle in cows diagnosed with COF [39].

GnRH was first reported in the early 1970s as an effective treatment for follicular cysts in cattle [40]. In this small early study, all treated cows ($n = 5$) were observed in standing estrus within 20–24 days following GnRH treatment [40]. Many subsequent studies have shown the value of treating cows with COF with GnRH products and its use in the management of COF continues today. Across many studies, there have been wide variations in the results observed in the treatment of COF with GnRH alone.

Following an injection of GnRH a surge of LH from the anterior pituitary occurs within two hours [17]. This LH surge acts to cause luteinization of the follicular cyst, which will then generally undergo spontaneous luteolysis in 18–20 days, followed by a normal estrous cycle. However, it is common for the injection of GnRH to cause ovulation of a dominant follicle also present on one of the ovaries at the time of treatment. With this situation, ovulation will occur as well as initiation of a new follicular wave, followed by a normal luteal phase [41]. The subsequent increase in progesterone concentrations from the luteinized cyst lasts for 15–18 days and allows the resetting of the normal hypothalamic–pituitary–ovarian axis, with resumption of the positive feedback of estradiol to the hypothalamus. This leads to resumption of normal cyclicity in 72–85% of cows, with a return to estrus generally within three weeks [2]. However, the interval from GnRH injection to estrus can be reduced with the use of prostaglandins administered 7–10 days after GnRH treatment, causing luteolysis and subsequent estrus.

In one study looking at the effectiveness of a single injection of GnRH for treatment of cows with COF, 72% of cows resumed normal cyclicity within 20 days of treatment compared with 16% of control cows [17]. However, as mentioned above, findings have not been consistent across studies. There was no difference in the period of time between treatment with GnRH alone and resolution of COF or in the period of time between treatment and a CL being observed [42]. These studies highlight the difficulty in evaluating the effectiveness of GnRH alone as therapy for COF because of the high number of cows that appear to recover spontaneously and the variability and confounding factors present in the numerous study designs.

No differences have been observed when comparing synthetic GnRH to GnRH analogs or among different concentrations of GnRH. The typical dose administered in the treatment of COF is 100 μg intramuscularly and any of the commercially available products should assure similar results [43, 44].

Human Chorionic Gonadotropin

Human chorionic gonadotropin (hCG) is a protein hormone that has been used to treat refractory cysts that fail to respond to GnRH. hCG has high LH-like properties that will often cause the cyst or other follicles present to luteinize and subsequently begin producing progesterone [45]. Once luteinized, the cyst can then be treated with prostaglandins to induce regression of the functional luteal structures and reinitiate normal estrous cycles. The most common dosage used is 10000 IU given intramuscularly. Its use has occasionally been noted to stimulate an immune reaction leading to refractoriness, although the importance of this reaction is poorly understood [1]. It is a more costly treatment than GnRH and it has been known to occasionally cause anaphylactic reaction following treatment. For these reasons, it is generally only used when GnRH or other therapies have failed to render a cure.

Progesterone

Use of progesterone as a treatment for COF was proposed over 40 years ago [46]. With the approval of progesterone-impregnated controlled intravaginal drug-releasing devices (CIDRs) for use in lactating dairy cattle in a number of

countries there have been many studies evaluating their effectiveness in the treatment of COF. Treatment with these CIDRs reduces LH secretion, results in regression of the cystic structure, and initiates a new follicular wave following removal of the implant. Progesterone has been shown to reestablish the normal feedback mechanisms involving the hypothalamic–pituitary–ovarian axis and allow cows with COF to resume normal cyclicity. The precise mechanism for how that occurs remains somewhat elusive, but the following model proposed by Todoroki et al. is the current hypothesis. Progesterone released from the CIDR raises circulating progesterone levels to those comparable with levels during the normal luteal phase. This heightened level of progesterone induces atresia of the cystic follicles by lowering the LH pulse frequency. This then allows for relief from the inhibitory effects of elevated estradiol and inhibin produced by the cystic follicle so that emergence of a new follicular wave can occur. Additionally, progesterone prevents excessive growth of the emerging dominant follicle and allows for turnover of follicles at normal intervals by suppressing LH secretion. Progesterone is believed to be able to restore the hypothalamic–pituitary–ovarian axis to produce an adequate LH surge in response to elevated estradiol production by the dominant follicle following removal of the progesterone source [16, 47–49]. The duration of progesterone treatment which is sufficient to reestablish the normal feedback mechanism involving the hypothalamic–pituitary–ovarian axis appears to be as short as three days [50].

Progesterone can be used as the sole treatment for COF. There were comparable pregnancy rates in cows with COF who were treated with the Ovsynch protocol and bred via artificial insemination (AI) versus cows who received a CIDR for seven days with a prostaglandin injection on removal and bred via AI after heat detection, suggesting that CIDRs alone can be effective in treating cows with COF [51]. CIDRs can also be used very effectively in the treatment of COF when combined with the Ovsynch protocol mentioned later in this chapter.

Todoroki et al. [47] studied the effects of using CIDRs for the treatment of superovulated beef-cow donors who were diagnosed with follicular cysts and found favorable results. Over a period of two years, 28 cows were diagnosed with COF and treated with a CIDR for 14 days. After removal of the CIDR all cows exhibited estrus behavior and ovulated between 2 and 10 days following CIDR removal and subsequently formed corpora lutea. In 18 of 28 (64%) treated cows that showed initial recovery, the COF condition did not recur after repeated embryo recovery attempts. However, the COF condition did recur in 10 of 28 (36%) cows after the next embryo recovery [47].

The use of CIDRs in place of traditional prostaglandin for synchronization of estrus after embryo recovery reduced the incidence of follicular cysts in superovulated beef-cow donors [47]. Immediately after embryo recovery, a CIDR was placed in the vagina of each cow and left for 14 days. Todoroki et al. [47] showed that when CIDRs were used in place of prostaglandins for synchronization of estrus after embryo recovery, there was a marked reduction in the number of cows subsequently diagnosed with COF. In year one of the study of 61 donors, of which 22 had a history of COF, only two developed COF. In year 2 of the study, among 54 donors, of which 17 had a history of COF, no cows developed COF. CIDRs proved effective both in the treatment of COF and in lowering the incidence of COF in beef-cow donors, where it was used in place of prostaglandin for synchronization of estrus after embryo recovery.

Progesterone clearly appears to have some healing effects on the functions of the hypothalamic–pituitary–ovarian axis in cows with COF [46–48]. The use of progesterone either alone or in conjunction with other hormonal therapy can be very effective in treatment of cows diagnosed with COF.

Prostaglandins

Prostaglandins are limited to the treatment of luteal cysts as their use has no effect on follicular cysts. Once a follicular cyst undergoes lutenization, it will become a luteal cyst and would then become responsive to prostaglandins [1, 2, 7, 41]. The ineffectiveness of prostaglandin use in the management of follicular cysts emphasizes the importance of accurate diagnosis prior to using prostaglandins as a single modality treatment option. Following prostaglandin treatment of luteal cysts, regression of the cyst occurs, with estrus occurring in 90% of cows by day 8 following treatment [2]. In cases where a determination of the exact cyst type is not made or cannot be determined before initiation of treatment, it is recommended to give prostaglandins concurrently with GnRH. This allows for the most efficient treatment of any cyst that is a luteal cyst while also appropriately treating all follicular cysts. It has also been shown that the simultaneous use of GnRH and prostaglandins at the initiation of the Ovsynch protocol in lactating dairy cows with COF showed improved pregnancy rates over the standard Ovsynch protocol and rates comparable to normal controls when heat detection and AI were used [9].

Ovsynch Protocol

Numerous protocols have been reported involving a series of hormonal injections aimed at treating COF and restoring the cow to normal cyclicity [1, 8, 51–54]. It has been shown that progesterone levels in cows with COF that were treated with a single injection of GnRH are elevated at five days following treatment. This indicates that the Ovsynch protocol can be

used with good results [52]. The classic Ovsynch protocol has been used in the treatment of cows with COF. Pregnancy rates observed following Ovsynch treatment of those cows varied from 17 to 25% [54, 55]. The rationale for and benefit of using the Ovsynch protocol to treat cows with COF is that it should work regardless of the specific cyst type and should provide an opportunity to inseminate the cow without the need for heat detection [8, 51–54]. This flexibility has allowed the Ovsynch protocol to gain popularity because it is familiar to many producers and because of its "one size fits all" usefulness regarding treatment of cows with COF regardless of the specific cyst type.

As mentioned in the section "Prostaglandins," combined treatment with GnRH and prostaglandin increased the number of cows with COF that responded appropriately to an injection of prostaglandin 14 days later. Utilizing both hormones at initiation of the Ovsynch protocol allows for any cow with luteal cysts to be treated in the most efficient manner possible.

Combining the Ovsynch protocol with a CIDR for treating cows with COF is very effective. This combination alters the endocrine state to allow recruitment of a new follicular wave, causing COF turnover and resetting the hypothalamic–pituitary axis, with the added benefit of allowing fixed-time artificial insemination (FTAI). The combination of the additional progesterone provided by the CIDR should assure all cows are exposed to its beneficial effects. Results have shown an increase in pregnancy rates in cows with COF that were treated with Ovsynch plus CIDR (37.5% pregnancy rate) over cows treated with Ovsynch alone (16.7% pregnancy rate) [53].

Additional Therapies

Two reports evaluating the effectiveness of the opioid antagonist naloxone as a treatment for COF have been published [56–57]. It has been shown that stress may be a contributing factor in the pathogenesis of COF in cattle. Endogenous opioid peptides are involved in many responses to stress, including the regulation of various endocrine systems [56]. Endogenous opioid peptides are believed to block the release of GnRH from the hypothalamus as well as the estrogen-induced LH surge [58]. It has been shown that administration of the opioid antagonist naloxone results in elevated LH release in cattle under various stressful states [59]. When naloxone was combined with an injection of the GnRH agonist buserelin in treatment of cows with COF, 77.5% of the treated cows had begun cystic regression, as viewed with ultrasonography, or had begun cycling normally within two weeks after treatment [56]. In a subsequent small study, cows with COF were treated with naloxone and demonstrated improved pregnancy rates (numerical), but the increase was not considered statistically significant [57]. Before this therapy can be recommended a much larger and statistically powerful study is warranted to evaluate naloxone as a treatment for COF in cattle.

Associations

Numerous risk factors have been associated with the formation of COF. The association between high milk production and the incidence of COF has been noted for over 50 years. Most studies show that there is either a positive association with increased milk production and the incidence of COF or no association with increased milk production and the incidence of COF. However, it should be noted that no study to date has shown that as milk production increases, the incidence of COF goes down. As cows are increasingly selected for high milk production and are pushed metabolically to meet their lactation potential, stressors associated with these demands contribute to the formation of COF. Whether the association between milk production and COF formation is a cause-and-effect relationship has not been clearly identified yet.

During early lactation, dry matter intake is often insufficient to meet the nutritional demands of lactation. The ensuing nutritional imbalance results in an increase in NEFA from the mobilization of adipose tissue to ultimately lead to production of ketones (primarily beta hydroxybutyrate), which serve as a key indicator of negative energy balance. High levels of NEFA have been shown to have a detrimental effect on follicular growth and appear to be involved in the development of COF [60]. The use of bovine somatotropin does not appear to be associated with any increased incidence of COF in dairy cattle [61, 62].

A study attempting to identify risk factors associated with postpartum COF formation showed that the condition was most commonly seen in cows calving in the summer months, higher-producing dairy cattle, older cattle, and cows that increased in body condition immediately preceding calving [9]. The presence of COF in the early postpartum period was the single greatest risk factor for the presence of COF at the time of insemination in dairy cattle. The risk of having COF at the time of insemination was 36.6% higher for cows that had developed COF early in the postpartum period [9]. These findings would seem to suggest that treatment of multiparous cows with COF early in the postpartum period would be beneficial, whereas younger cows could be delayed until the end of the voluntary waiting period to allow for spontaneous resolution [9]. Dairy cows that were identified as having had an abnormal puerperium were 1.9 times more likely to develop COF in the postpartum period. Also, cattle that had a 1-unit increase

in body condition score in the prepartum period were 8.4 times as likely to develop COF [8]. Dairy cows that deliver twins were 2.0–2.7 times more likely to develop COF in the postpartum period compared with cows that did not deliver twins, suggesting that delivery of twins may predispose the cow to COF in the subsequent lactation [63, 64].

Metabolic factors that have been shown to be associated with increase in the incidence of COF include overconditioned cattle, reduction in insulin and IGF-1, and increased non-esterified fatty acids [5, 7]. Recent studies have proposed that imbalances in hormones such as insulin, leptin, and adiponectin as well as several metabolites such as glucose, non-esterfied fatty acids (NEFA), and some amino acids have been put forward [65]. Additional factors that have been shown to be associated with COF formation but without a clear causal relationship include heat stress, utilization of bulls with high genetic merit for fat and protein, high stocking rates in barns, and melengestrol acetate use in beef heifers [66, 67].

There is a genetic predisposition for COF in dairy cattle, although the heritability is low at 0.07–0.12 [68, 69]. Certain cow families in the dairy breeds appear to have a higher incidence of COF than their contemporaries. Swedish workers showed that when sires were removed from the breeding pool after producing daughters that developed COF, they could significantly reduce the incidence of COF from 10 to 3% [70]. An aspirational goal would be to select cow families both with high milk production and that show little genetic predisposition to the development of COF. Genetic selection as a means of prevention of COF can be effective. One must weigh the impact of such selection, as often milk production will be selected against as well.

Summary

Efforts to reduce the incidence of common postpartum diseases and stress should be the focus of prevention strategies. Prompt diagnosis with the use of ultrasound should allow for early recognition and the selection of effective treatments. The simultaneous use of GnRH and a prostaglandin should be considered especially when the type of cyst is unknown, as this combination allows for efficient treatment regardless of the cyst type. Efforts moving forward should be focused on minimizing the contributing factors that have been associated with COF formation and treating cows in a prompt time frame to assure they can be available for breeding in a timely manner to avoid the economic losses associated with this condition.

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34

Postpartum Anestrus and Its Management in Dairy Cattle

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Introduction

Postpartum anestrus refers to a condition where cows have not been observed or reported in estrus for several weeks after calving, often to the end of the voluntary (elective) waiting period, in dairy cattle. While a short period of ovarian inactivity during the immediate postpartum period is normal, extended anestrus in non-suckled cattle such as dairy cows could have negative ramifications on the timely reestablishment of pregnancy [1–4]. Postpartum anestrus has been recognized as a problem by animal scientists for several decades. According to one source [5], anestrus in cattle was first reported as a reproductive problem back in the 1920s [6]. There has been significant interest in the past two to three decades in the understanding and management of postpartum anestrus, both in beef and dairy cows [5, 7–14]. Nevertheless, postpartum anestrus continues to be a challenge to dairy practitioners and farmers alike. This chapter will focus primarily on the problem of postpartum anestrus and its management in dairy cattle, although references to the condition in beef cattle will be made where appropriate.

Fertility of the High-Producing Dairy Cow

The modern dairy cow is an important livestock species with a remarkable ability to produce tens of thousands of liters of milk during her lifetime. Understandably, the profitability of dairy farming largely depends on the reproductive success of dairy herds [15, 16]. However, suboptimal fertility of the modern dairy cow is a problem that afflicts the dairy industry worldwide [17–21], with reproductive failure often being the primary reason for culling dairy cows [22–24]. While the reasons for declining fertility in dairy cattle will remain debatable, it is important to bear in mind that the modern dairy cow is physiologically quite different from her counterparts of past decades [19], calling for new and improved strategies of reproductive management to maintain or enhance reproductive success. An extended period of postpartum anestrus is one among the many factors contributing to poor reproductive efficiency in dairy cattle. Rajala-Schultz and Gröhn [25] showed that if a cow had not been inseminated at all, her risk of culling was 10 times higher than if she had been inseminated at least once. While a deliberate managerial decision to not breed a cow, due to past history, may have directly contributed to this increased culling risk, anestrus could also have increased the risk, emphasizing the importance of detecting cows in estrus and having them bred in a timely manner.

Prevalence and Implications of Postpartum Anestrus

The prevalence of postpartum anestrus in dairy cattle is herd-specific and varies widely from one herd to another. However, typical prevalence rates at the end of the elective waiting period (60–80 days after calving) are in the range 10–30% [26–31], whereas higher rates of up to 59% have been reported in individual herds [19, 28, 30]. In a Canadian study involving 23 herds, milk progesterone concentrations were measured twice weekly in 637 cows to determine the onset of cyclicity, starting approximately from 7 to 90 days after calving [31]. The overall mean interval from calving to first rise in progesterone, indicative of an ovulation, was 32.0 ± 0.7 days. The cumulative percentages of cows initiating cyclicity by 3, 6, 9, and >9 weeks after calving are shown in Figure 34.1.

In a more recent study [32] on 748 Canadian Holsteins from two herds, commencement of luteal activity was determined based on milk progesterone concentrations

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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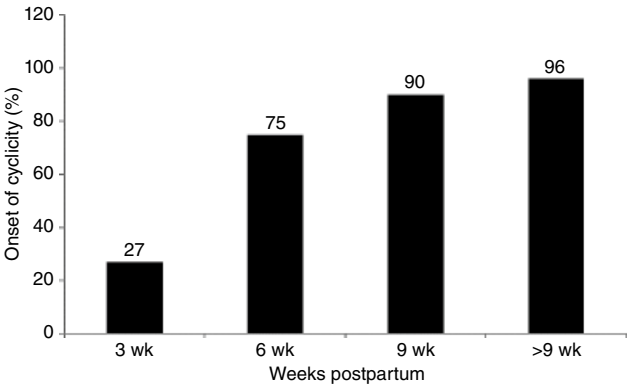


Figure 34.1 Onset of cyclicity (data presented as cumulative percentages) in 637 dairy cows (23 herds) by 3, 6, 9, and >9 weeks postpartum determined based on milk progesterone concentrations quantified twice weekly from 7 up to 90 days postpartum or until first service, whichever occurred earlier. Mean interval from calving to first rise in progesterone (indicative of first ovulation) was 32.0 ± 0.7 days (range 5–113 days). *Source:* [31].

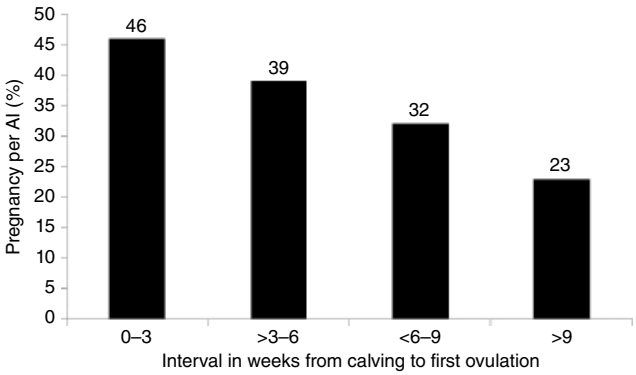


Figure 34.2 P/AI to first service by interval from calving to first ovulation in 637 cows (23 herds) determined based on rise in milk progesterone concentrations quantified twice weekly from 7 up to 90 days postpartum or until first service, whichever occurred earlier. The first rise in progesterone was indicative of ovulation. Cows that had the first rise in progesterone within three weeks after calving had the highest ($P < 0.03$) P/AI to first service. *Source:* [31].

using an automated in-line milk analysis system (Herd Navigator™, DeLaval International, Tumba, Sweden). The proportions of cows determined to have commenced luteal activity earlier than 4, 5, 6, 7, 8, and 9 weeks postpartum were 27, 53, 68, 77, 84, and 89% (cumulative percentages) in the aforesaid study, respectively. Although these collective findings suggest that postpartum anestrus is not necessarily a huge problem because approximately 90% of the cows had initiated cyclicity by 9 weeks – the typical end of the elective waiting period – it is important to note that cows that initiated cyclicity during the early postpartum period had higher pregnancy per artificial insemination (P/AI) at first service (Figure 34.2). Similar findings have been reported by others [1, 2, 4, 27]. Bruinjé et al. [32] found that the proportion of cows commencing luteal activity later than 28 days postpartum was greater among primiparous than multiparous cows (79.1 vs 68.6%; $P < 0.01$); the same trend was evident later in lactation when more primiparous than multiparous cows (13.1 vs 8.3; $P < 0.05$) remained acyclic beyond 63 days postpartum, indicating the influence of parity on anestrus.

The biggest impact of postpartum anestrus is the extension of the interval from calving to conception, which consequently impacts the calving interval. Whereas high-producing dairy cows that fail to return to estrus and conceive soon after the end of the elective waiting period may continue to remain profitable for a period of time, cows with lower production that remain acyclic for an extended period, resulting in delayed conception, are often unprofitable and become potential targets for culling.

Physiological Basis for Postpartum Anestrus and Resumption of Cyclicity

Endocrine Events Leading to Anestrus

During gestation, circulating progesterone and estrogen concentrations remain high, exerting a suppressive effect on the hypothalamic–pituitary axis [33]. Although peripheral progesterone concentrations in late gestation are not much higher than that of mid-luteal or early gestational periods [34, 35], circulating estrogens progressively increase, starting at about 60 days of gestation and reaching 50- to 1000-fold higher concentrations in the days immediately preceding parturition [34, 36, 37]. The sustained long exposure to high concentrations of estradiol, either by itself or in tandem with progesterone, inhibits hypothalamic gonadotropin-releasing hormone (GnRH) secretion, which negatively affects the synthesis of luteinizing hormone (LH), leading to a gradual depletion of pituitary LH reserves. Although the mechanisms for release of LH from the anterior pituitary gland remain intact and functional throughout pregnancy [33], the absence of pulsatile GnRH secretion from the hypothalamus is what appears to negatively impact LH production.

Unlike LH, changes in the concentrations of follicle stimulating hormone (FSH) during late gestation are less remarkable. While mean FSH concentrations do not differ between prepartum and postpartum periods [34, 38], the recurrent rise (surge) and fall of FSH that typically occurs every few days in cyclic and pregnant cows is largely absent [34] or slowed down [38] during the last weeks preceding parturition. Consequently, the associated

follicular wave emergence is also absent during the last 30 days before parturition [38].

Whereas it is possible to restore the pulsatile release of LH under experimental conditions by administering low-dose GnRH injections at frequent intervals (one to two hours), not all cows respond to such treatment. Because of the inconsistent response to such an approach, it is believed that the inhibition of GnRH secretion is perhaps not the only mechanism involved in anestrus [5].

Endocrine Events Leading to the Resumption of Cyclicity

As parturition approaches there is a gradual decrease in progesterone concentrations. In contrast, estrogens keep rising and attain peak concentrations during the last 24 hours prior to parturition [36, 37]. Since almost all circulating estrogens are of placental origin, their concentrations plummet sharply after expulsion of the conceptus, reaching basal levels within one day after calving [36, 37]. In the absence of progesterone and estradiol in the immediate postpartum period, the hypothalamic–pituitary axis is "liberated" from the suppressive effects of these steroids, resulting in the restoration of normal function of the GnRH pulse generator and the resultant pulsatile release of GnRH, which acts on the pituitary to restore a normal pattern of FSH release. Normal FSH surges resume within three days after calving, triggering the emergence of a follicular wave [34]. Whereas early stages of follicle development are gonadotropin independent, FSH support is required for the growth of follicles from 4 to 9 mm and LH pulses are indispensable for continued growth of follicles beyond 9 mm in diameter [39, 40].

During the early postpartum period the LH pulse frequency remains very low, generally less than one pulse every four hours, which later increases to about one to two pulses per hour as GnRH pulsatility increases. The restoration of pituitary function and reaccumulation of LH could take up to three weeks or longer depending on several factors. Thus, when conditions are optimal for pituitary LH reserves to be replenished, normal LH pulsatility is restored, leading to the growth and establishment of dominant follicles capable of producing greater quantities of estradiol. A high concentration of estradiol from the dominant follicle is essential to trigger an LH surge, which is critical for the first ovulation to occur. Although the first ovulation signals resumption of cyclicity, not all cows would resume normal ovarian cycles following the first ovulation; at least some cases could return to anestrus [41].

If cows are challenged with either exogenous GnRH or estradiol during the early postpartum period, not only is LH release greatly diminished due to lack of pituitary LH reserves, but also its bioactivity is lowered [5]. Kisspeptin – a more recently discovered neuroendocrine regulator of reproduction – controls GnRH synthesis in the hypothalamus, and subsequently influences LH release in many species including cattle [42]. Exogenous administration of kisspeptin induced ovulation in seasonally anestrous ewes [43] and both GnRH and LH responses to kisspeptin were greater in anestrous ewes than in cyclic ewes during luteal phase [44]. Although little is known about the direct role of kisspeptin in the cessation of postpartum anestrus in cattle, at least one study has presented evidence to suggest that kisspeptin plays a key role in the resumption of cyclicity after pregnancy-induced anestrus in dairy cows [45].

Fate of the First Dominant Follicle

The fate of the first dominant follicle in the postpartum cow is determined by the pulsatile LH support it receives and by its own steroidogenic capacity to produce estradiol. Depending on these factors, the first dominant follicle may (i) ovulate and form a corpus luteum (CL); (ii) become anovulatory, possibly developing into a cystic follicle; or (iii) regress, making way for a new dominant follicle [46, 47].

Classification of Postpartum Anestrus

Postpartum anestrus can be classified into different types based on either ovarian follicle dynamics or progesterone profiles.

Categorizing Anestrus Based on Ovarian Follicular Dynamics

The classification of anovulatory conditions in cattle based on three functionally critical follicular diameters relating to emergence (~4 mm), deviation (~9 mm), and ovulation (10–20 mm) has been proposed [48]. With the above classification as the basis, four types of anestrus, types I to IV, were described by Peter et al. [13] (Figure 34.3).

In type I anestrus, there is growth of follicles up to emergence but no deviation occurs, resulting in lack of selection of a dominant follicle. This type of anestrus is presumed due to extreme malnutrition, which could exert a negative effect on FSH production, suppressing follicular growth, although other factors could also be involved. Ovaries under this type may be described as either "inactive" or "smooth" as a reference to the lack of palpable ovarian structures during reproductive examinations per rectum. An Israeli study [49] from the 1980s reported an incidence of 8.5% inactive ovaries from 7751 lactations. While type I anestrus is not widely seen in well-nourished dairy cattle in

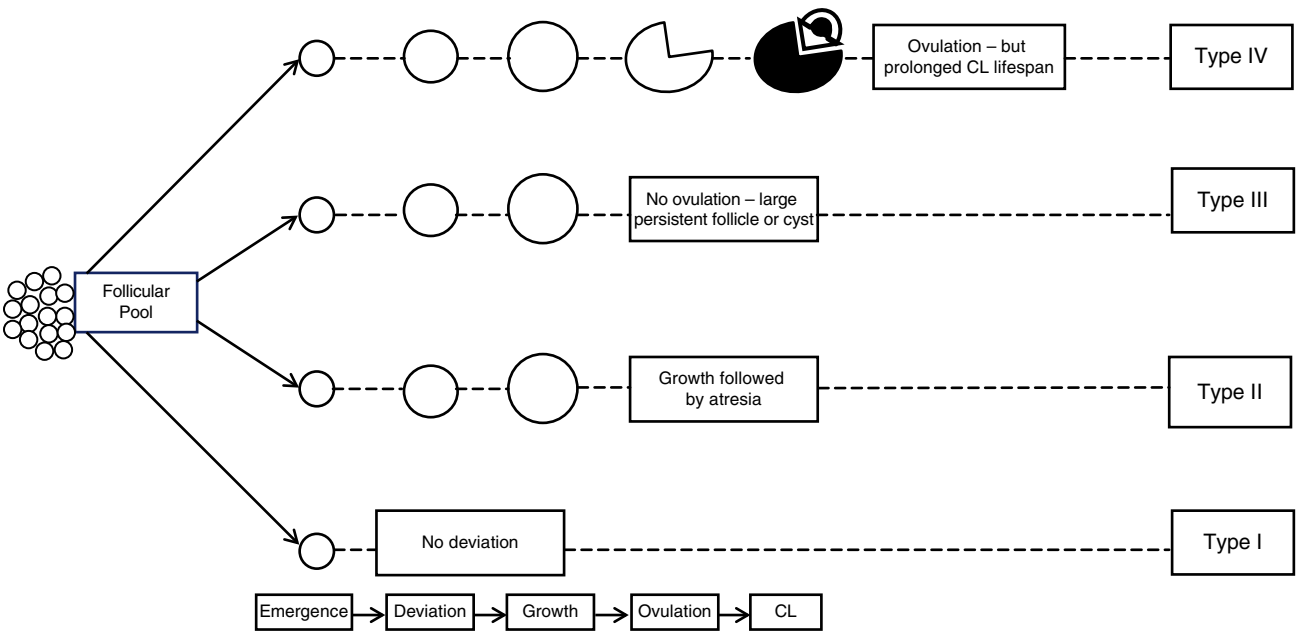


Figure 34.3 Schematic representation of types of anestrous conditions based on ovarian follicular dynamics. *Source:* Modified from [13].

the USA and Canada, this type of anestrus could be more prevalent in regions of the world where balanced energy-dense rations are unavailable to dairy cattle.

In type II anestrus, both follicular deviation and growth occur, followed by regression, in some cases, after a follicle attained dominance. Regression of a dominant follicle is usually followed by the emergence of a new follicular wave, two to three days later. In this type of anestrus, there may be sequential emergence of follicular waves prior to the eventual occurrence of the first ovulation. Up to nine waves of follicular growth have been reported in one study [50].

In type III anestrus, deviation, growth, and establishment of a dominant follicle takes place, but the dominant follicle fails to ovulate, becoming a persistent structure which may either linger as an anovular follicle or continue to grow and develop into a cystic follicle. Anovular follicles are considered different from cystic follicles because in cows with the latter condition there is disruption of the feedback mechanisms in the hypothalamic–pituitary axis [51]. The secretory pattern of LH in cows with cystic ovarian follicle is quite different from that in normal cyclic cows in that mean concentrations of LH, frequency of LH pulses, and amplitude of LH pulses are all higher [52]; in addition, in cows with an actively growing cystic follicle, the estradiol concentration is significantly higher [53, 54]. A cystic follicle that is growing actively usually exerts dominance, suppressing the growth of other follicles. Upon retrospective analysis of data obtained from twice-weekly ovarian ultrasonography (7–35 days after calving), on eight lactating dairy cows that developed cystic ovarian follicles, a first-wave dominant follicle became cystic in seven cows, and a second-wave dominant follicle became cystic in one cow. No other follicle greater than about 5–6 mm diameter was detected on either ovary of cows until the cystic follicle turned over. In five cows, the cyst persisted until (possibly beyond) day 35; in two cows, the initial cystic follicle was replaced by another one; in one cow two follicles from the second follicular wave became dominant around day 30, at which time the original cyst had regressed under 20 mm diameter, giving way for both dominant follicles to ovulate (Divakar J. Ambrose, personal observations).

In type IV anestrus, a dominant follicle ovulates and forms a CL, but the luteal phase is prolonged due to the absence of timely luteolysis followed by CL regression. Aberrant follicular growth patterns resulting in the absence of an estrogenic dominant follicle at the ideal time to trigger luteolysis could be a reason for this condition of persistent CL [48].

Categorizing Anestrus Based on Progesterone Profiles

Cows can also be classified into different categories of anestrus based on progesterone measured in milk or blood at least on a twice-weekly basis. Using twice-weekly measurement of progesterone in milk fat on 334 dairy cows from six herds, Opsomer et al. [55] reported that 51% of the cows had a normal progesterone profile with the first rise in progesterone occurring before 50 days after calving, followed by regular cyclicity. Among the remaining popula-

tion, 21.5% of the cows had delayed cyclicity (anestrus for the first 50 days after calving, characterized by consistently low progesterone during that entire period), 21.5% had prolonged luteal phase (progesterone remained elevated for >20 days without a preceding insemination), 4% had cessation of cyclicity (normal cycles interrupted by at least 14 days of consistently low progesterone concentrations), 0.5% had short luteal phases (characterized by more than one luteal phase, excluding the first luteal phase, that was shorter than 10 days' duration), and 1.5% of the cows had irregular progesterone profiles (atypical profiles, that did not belong to any of the previously described categories).

Factors Contributing to Postpartum Anestrus

The causes of postpartum anestrus in cattle are often multifactorial [5, 8, 10, 12, 13, 47, 56–60] but can be broadly categorized into physiological, nutritional, managerial, environmental, and pathological, as detailed in Table 34.1 (and Figure 34.4). Among the numerous potential contributors, suckling and nutrition (negative energy balance) are considered the most important.

Suckling

Suckling is a major contributor to anestrus in beef cattle [56], but it is generally not considered a cause for anestrus in dairy cattle because of the prevailing industry practice of separating dairy calves from the dam soon after birth.

However, under experimental conditions when identical twin pairs of dairy cows were assigned to either suckling by multiple calves or twice-daily machine milking, the suckled cows had a five- to nine-week longer interval to first estrus after calving than their machine-milked twins. Basal plasma concentrations of LH did not differ between suckled and machine-milked twins, yet GnRH-induced LH release increased progressively in machine-milked cows from weeks zero to six after calving, and the LH response at two weeks after calving was significantly higher in machine-milked cows than in their suckled twins [90]. Estradiol-17β-induced LH release within 24 hours after treatment was delayed by up to 6 weeks in suckled cows compared with their machine-milked twins, indicating that the response of the hypothalamic–pituitary axis to both GnRH and estradiol-17β is impaired by suckling. In another study [91], suckled dairy cows had lower mean LH

Table 34.1 Direct and indirect causes of anestrus in cattle.[1]

Causal factor	Comment
Physiological	
Suckling	Suckling suppresses episodic LH secretion, thereby delaying ovulation [56, 59, 61]. Suckling is a major contributor to postpartum anestrus in beef cattle, but not so in dairy cattle because dairy calves are usually separated from the dam soon after birth
Parity	Parity as a risk factor for anestrus has been reported in numerous studies [32, 55, 57, 62]. Most reports suggest that first-parity cows are at a higher risk of anestrus due to delayed first ovulation. Multiparous cows, particularly those with four or more calvings, are reportedly at a significantly higher risk for anestrus due to prolonged luteal phases. The reason may be that with increasing parity the uterus takes more time to return to its pregravid size, thereby increasing the risk of pyometra and persistent CL [55]
Milk yield	Although high milk production has been identified as a risk factor for anestrus by some researchers [30, 62, 63], others have not found such an association. In one study [30], the probability of anovulation decreased by 2% for every 100-kg increase in the first 305-day mature-equivalent milk projection between 5000 and 9500 kg, and the risk of anovulation did not change as first 305-day mature-equivalent milk production increased above 9500 kg. Similarly, another large study [62] found that cows with lower daily average milk yield in the first 90 days postpartum (32.1 vs 39.1, 43.6, 50.0 kg/day) were at higher risk for anestrus. It is unlikely that high milk production itself is a cause for anestrus; however, the inability to meet energy requirements leading to negative energy balance, a common problem in lactating dairy cows, is the most likely reason
Ratio of increase in milk yield	The ratio of increase in milk yield from the first week postpartum to the week when milk yield reached its peak is reportedly smaller in ovular than in anovular cows [64]
Social or chemical cues	In beef cattle, the interval from calving to resumption of cyclicity is significantly reduced by the presence of bulls [65]. In one study, the exposure to bulls in the early postpartum period had an acute effect on LH release in anestrous dairy cows [66]. Likewise, the presence of other estrous females or exposure to cervical mucus collected from estrous cows can shorten the duration of postpartum anestrus [67], particularly in cows with extended anestrous periods

Table 34.1 (Continued)

Causal factor	Comment
Breed	The influence of breed or type (genetic) differences on the interval from calving to commencement of luteal activity has been reported in dairy [68], beef [57], and crossbred cattle [68, 69]
Nutritional	
Malnutrition	Severe feed restriction will induce anestrus in cattle. In one study [70], non-lactating beef cows placed on a restricted diet formulated for 1% of body weight loss each week became anestrous after 26 weeks and lost 24% of their initial body weight. Estrous cycles resumed about 9 weeks after cows were returned to a diet that was 160% of the maintenance diet given to control cows. Anestrus was associated with a decrease in the frequency of LH pulses
Negative energy balance	Cows in negative energy balance are at a very high risk of anestrus and delayed onset of cyclicity [11]. Elevated circulating concentrations of non-esterified fatty acids are associated with adipose tissue mobilization and indicative of negative energy balance
Body condition and rate of loss of body condition	A body condition score (BCS) of less than 3.0 on a 1–5 scale is commonly an indicator of negative energy balance [55, 62]. Cows with low BCS are more likely to be anestrous and it has been reported that the influence of body condition on the duration of postpartum anestrous period is mediated through differences in LH pulse frequency [71]. Cyclic cows with low BCS tend to have poor P/AI and higher embryonic losses [62, 72, 73]. A higher rate of body condition loss has been associated with extended intervals from calving to resumption of cyclicity or first service [74–76] (Figure 34.4)
Managerial	
Length of previous dry period	Cows having a dry period longer than 77 days were almost three times more at risk of anestrus and delayed ovulation [55]. In contrast, the interval from calving to first ovulation in cows given no dry period (fed a high-energy diet continuously) compared to those given a 56-day dry period (fed a low-energy diet from 56 to 29 days prepartum followed by a moderate-energy diet for the last 28 days) was significantly shorter (13.2 vs 31.9 days) [77, 78]
Increased milking frequency	Increasing the frequency of milking from twice to three or four times daily lengthens the interval from calving to first ovulation by less than a week. When milking frequency is increased from three to six times daily, the interval to first ovulation is lengthened, but still not to the same extent as that of frequent suckling [59]. Therefore increased milking frequency is only a minor contributor to anestrus
Efficiency of estrus detection	Unobserved estrus due to poor estrus detection efficiency can frequently contribute to anestrus [31], exacerbating the severity of the condition
Feed and feeding management	Inadequate dry matter intake is one of major reasons for cows entering a state of negative energy balance [47, 79, 80]. Maximizing dry matter intake through particle size management, frequency of feed delivery (more than one feed), grouping cows by parity/production levels/social order, feed bunk management (e.g. frequent push-ups), discouraging sorting behavior by adding water, and avoiding overstocking are some examples of strategies to improve dry matter intake, thereby reducing negative energy balance
Environmental	
Calving season	Cows calving in the winter have delayed resumption of estrous cycles compared with those calving in the summer [7, 30, 55]. While some of the variations attributed to season are likely due to seasonal differences in management, photoperiod does seem to play a role in the regulation of postpartum cyclicity
Weather	Under extreme hot and humid conditions, cows have reduced expression of estrus [7], which contributes to an increase in anestrus due to subestrus and silent estrus
Social hierarchy and stocking density	Social hierarchy (e.g. presence of dominant cows) could be a factor that has detrimental effects on feed intake, particularly in overstocked barns. It is not uncommon to see "bullying" among cows. The subordinate or the "bullied" cows frequently end up eating leftover feed which may be of poor quality as it is likely to have been sorted through. Such cows are at higher risk for negative energy balance, and thus anestrus [81]

(*Continued*)

Table 34.1 (Continued)

Causal factor	Comment
Housing type	Cows under intensive housing systems compared with those on pasture have a higher risk of anestrus. Tie-stalled cows are more likely to be declared anestrus than cows in other types of housing [57]; one reason is that lactating cows housed in tie-stalls fail to manifest activity changes during estrus [82], likely increasing anestrus due to unexpressed or unobserved estrus
Stall design (cow comfort)	Poorly designed stalls (e.g. lack of lunge space) would limit the frequency of accessing feed bunk/manger, negatively affecting dry matter intake, predisposing cows to negative energy balance and consequently anestrus
Stray voltage	Few studies have examined the effects of stray voltage on reproductive function in cattle. Although no direct evidence of stray voltage on impairment of ovarian function could be found, it is a potential contributor to anestrus, as bothersome stray voltage in and around the feeding area could be a deterrent to feed intake
Pathological	
Uterine inflammation	Cows with acute forms of metritis and those with severe bacterial contamination of the uterus have delayed resumption of cyclic ovarian activity [55, 83]. The mean interval from calving to first ovulation was longer (45 vs 32 days) in cows that were determined to have uterine inflammation (based on higher proportion of polymorphonuclear [PMN] cells at endometrial cytology performed on day 25 postpartum) compared with cows with no uterine inflammation [84]. The cumulative pregnancy (percentage) at 270 days postpartum tended to be lower in cows with high PMN (58 vs 80) than in those with low PMN counts
Persistent corpus luteum	Cows with uterine infections accompanied by abnormal vaginal discharge are at much higher risk for anestrus due to prolonged luteal cycles [55]. However, prolonged luteal phases of >30 days' duration have been reported in cows that were not previously inseminated, even in the absence of any uterine infections [18, 85, 86]
Cystic ovarian follicle (COF)	Though not strictly a pathological condition, cows diagnosed with COF are often anestrous and anovulatory [87]. The precise etiology of COF is still not fully understood, but an aberration in LH secretory pattern is believed to trigger the formation of COF; cows with active COF have increased LH pulsatility, which is essential for continued growth of the cyst [51, 54]
Calving disorders	Dystocia, twinning, and retained placenta could predispose cows to anestrus [30, 55]
Metabolic disorders	Ketosis and displaced abomasum have been associated with delayed cyclicity and anovulation [30, 55], particularly in cows with subclinical ketosis during the first week postpartum [30]
Lameness	The increased risk of anestrus associated with lameness has been reported by many studies [55, 88, 89]
Mastitis	Mastitis by itself has been identified as a risk factor for anestrus [55]. Cows that suffered lameness and mastitis, or lameness and another severe stressor, were at much greater risk for delayed onset of cyclicity [89] than cows with mastitis alone
Other disease	Cows that developed pneumonia during the first month after calving were 5.4 times more at risk for delayed resumption of ovarian activity [55]. Numerous other types of systemic illnesses can contribute to the condition, as sick cows will have reduced dry matter intake, increasing their risk to enter a state of negative energy balance, and thereby anestrus

[1] This list should not be deemed complete and the references are representative citations only.

concentrations than non-suckled controls on day 13 after calving. This decrease resulted from a 60% reduction in the frequency and a 40% reduction in the amplitude of LH pulses. These authors concluded that decreased frequency and amplitude of episodic LH secretion and reduced capacity of pituitaries to respond to GnRH may account for suckling-induced inhibition of postpartum ovulation. For further reading on the effects of suckling on postpartum anestrus in cattle, please refer to reviews on the topic [56, 59, 60] and Chapter 24.

Nutrition

Inadequate consumption of dry matter (i.e. energy) resulting in negative energy balance is an important contributing factor to anestrus in dairy cattle [11, 47, 58]. Soon after calving, the energy needs of the newly lactating cow increase dramatically due to the higher energy requirements associated with milk production, pushing the cow into a catabolic metabolism. Energy balance is the net result of energy intake minus energy expended for maintenance, growth, and milk yield. Although dairy cows rarely enter into a state of

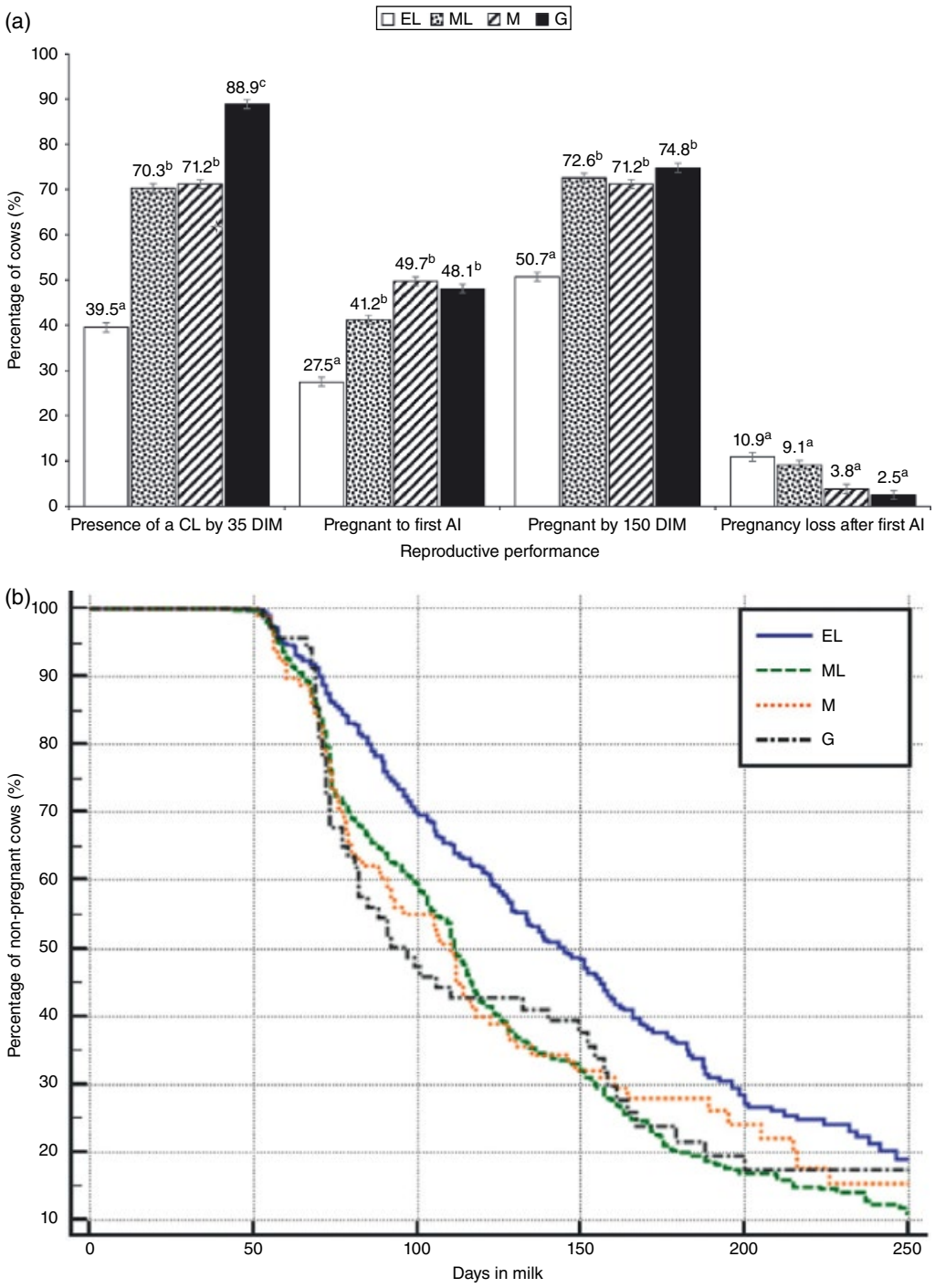


Figure 34.4 Results of a study of 911 lactating dairy cows assessed for BCS on a 5-point scale (1 = thin, 5 = fat) with 0.25-unit increments, at approximately 10 days precalving and 35 days postcalving, and categorized according to BCS change between the two time points. Categories were: lost ≥ 0.75 points (extreme loss: EL; open bars), lost either 0.25 or 0.50 units (moderate loss: ML; dotted bars), no change in BCS (maintained: M; hatched bars), or gained ⩾0.25 units (gained: G; filled bars). (a) Association between postpartum BCS change categories and reproductive outcomes. Transrectal ultrasonography of ovaries was performed at 34.6 ± 4.0 days in milk (DIM) to determine presence of a CL at 30–35 days postinsemination to diagnose pregnancy, and cows pregnant at first examination were reexamined 3–5 weeks later to confirm pregnancy. Pregnancy loss was declared when a cow diagnosed pregnant at first examination was not pregnant at reexamination. Different superscripts within same reproductive performance cluster differed (a, b, c; $P < 0.05$). (b) Kaplen-Meier survival curve showing probability of pregnancy up to 250 DIM by postpartum BCS change categories. Overall hazard of pregnancy differed ($P < 0.01$) between postpartum BCS change categories. Hazard ratio of pregnancy was 0.65 (approximately 35% lower pregnancy risk) for cows in EL category compared with ML category ($P < 0.01$), indicating fewer cows that had extreme loss of BCS were pregnant at 250 DIM compared to those that had moderate loss. Hazard of pregnancy did not differ between cows in ML, M, and G categories. *Source:* From [76], © 2019, Elsevier.

negative energy balance during the dry period, except in the immediate prepartum period when dry matter intake declines, most lactating dairy cows are unable to meet their energy demands after calving and invariably become energy deficient. Physical fill in the rumen is one of the factors limiting maximum dry matter intake in early postpartum dairy cows. Even if dry matter intake is maximized, cows selected for high milk production (e.g. North American Holstein) selectively use all available nutrients for milk production at the expense of body condition [80]. Thus nutrient partitioning prevents cows from ending the catabolic state because all the available glucose is preferentially used up for milk synthesis [92]. Despite the advances made in the understanding of dairy cow nutrition and the availability of high-precision ration-balancing software, meeting all the energy needs of a lactating dairy cow continues to remain a challenge, placing cows in a state of negative energy balance that extends for 10–12 weeks into the postpartum period [11].

Dairy cows that are in negative energy balance have lower blood concentrations of insulin and insulin-like growth factor (IGF)-1. Low circulating concentrations of IGF-1 reduce the negative feedback on growth hormone (GH), resulting in an increase in GH concentrations.

Increased GH increases liver gluconeogenesis and promotes lipolysis (mobilization of adipose tissue), resulting in the release of non-esterified fatty acids (NEFAs). High concentrations of GH and NEFA antagonize insulin action, creating a state of insulin resistance in postpartum dairy cows [79, 92], which diminishes glucose utilization by non-mammary tissues, conserving glucose for milk synthesis. Negative energy balance is strongly associated with the postpartum anovulatory period through decreased LH pulse frequency and low concentrations of blood glucose, insulin, and IGF-1 that collectively limit estrogen production by dominant follicles [11] which is essential for the LH surge, thereby delaying ovulations.

High stocking density that increases competition for feed, poor stall design that discourage cows from getting up frequently to eat, physical barriers that limit free and continuous access to feed, infrequent feed push-ups, excessive sorting by cows that get to the feed first, and inadequate hoof care leading to high prevalence of lameness are indirect causes that can reduce dry matter intake. Furthermore, social hierarchy situations where younger or subordinate animals are intimidated by herdmates, stray voltage, slippery footing, and other deterrents can dissuade cows from comfortably accessing feed, limiting dry matter intake and predisposing cows to enter a state of negative energy balance.

Unobserved Estrus or Silent Estrus

Unobserved estrus is often a major contributor to anestrus, as many of the cows considered anestrous could in fact be cycling normally, yet are never seen or reported in estrus. There are two categories of animals:

1) Subestrus: cows that express poor estrous behavior (e.g. short-duration, low-intensity estrus) and get missed during routine estrus detection.
2) Silent estrus: cows that fail to express any overt signs of estrus.

Both categories of cows may ovulate spontaneously and develop a CL; however, because they were never seen in estrus, they are falsely categorized as "anestrus." Performing more frequent visual detection of estrus (say three to four daily periods of dedicated surveillance) and observing for secondary signs of estrus such as chin-resting, vulva sniffing, licking, tailgating, and partial attempts to mount, rather than strictly for "standing estrus," will increase the probability of detecting estrus in these cows. The first ovulation after calving is rarely preceded by overt estrous behavior; only 10–13% of dairy cows reportedly exhibit standing estrus prior to the first ovulation [41, 93], although this proportion increases significantly in the second and third ovulatory cycles (Figure 34.5).

The level of milk production could also influence the proportion of cows detected in standing estrus at first ovulation [41, 93, 94], with a higher proportion of low-producing cows detected in standing estrus at first ovulation (Figure 34.6).

As previously stated, cows that initiate cyclicity earlier in the postpartum period have better reproductive performance [1, 2, 4, 27, 31, 32]. Therefore it becomes extremely important to assess the ovarian status of postpartum cows at regular intervals to differentiate cases of "anestrus due to unobserved estrus" from those that are "true anestrus." This is discussed in more detail in the section "Managing Anestrus."

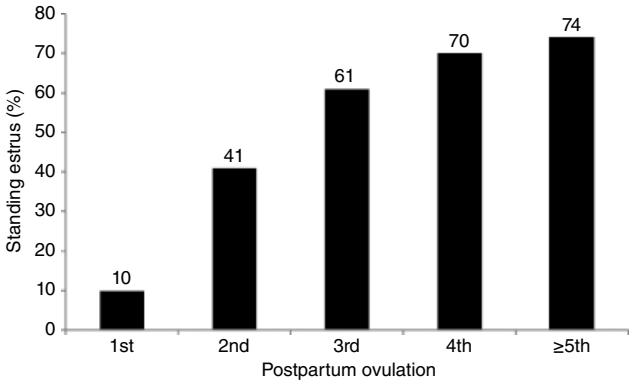


Figure 34.5 Frequency distribution of standing estrus behavior associated with first through fifth or greater postpartum ovulation in dairy cows. Standing estrus was associated with only 10% of first ovulations, but increased progressively at each subsequent ovulation. *Source:* Redrawn from [41].

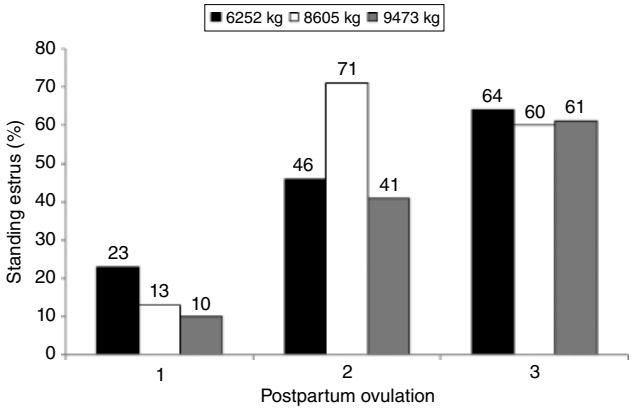


Figure 34.6 Frequency of occurrence of standing estrus and its association with level of milk production at first, second, and third postpartum ovulations. Percentage of standing estrus accompanying first ovulation was higher in cows with lowest annual production level of 6252 kg compared to those with higher production levels. Incidence of standing estrus at second and third ovulations increased substantially in higher-producing cows compared to that at first ovulation. *Sources:* Data are from references [41, 93, 94] for annual milk production levels of 9473, 8605, and 6252 kg, respectively.

Pregnancy

The primary physiological reason for cows being anestrus is the interruption of cyclicity by pregnancy. Although most dairy farmers keep excellent breeding records, it is not uncommon for errors to occur at the time of updating breeding records. The inadvertent transposition of numeric characters during data entry is a common error that can falsely identify inseminated cows as not inseminated, resulting in the occasional case of anestrus due to a "surprise pregnancy." During reproductive examinations, practitioners should always bear this in mind and rule out pregnancy first.

Managing Anestrus

Before planning a course of action to manage anestrus, it is important to assess the prevalence of anestrus at herd level. Anestrous postpartum beef cows, if treated, can recover from anestrus with normal fertility [95], whereas dairy cows treated for anestrus have much lower fertility [96], possibly due to an associated negative energy balance. Therefore every effort should be taken to return the cows to positive energy balance before first insemination.

General Strategies to Diagnose and Minimize the Impact of Anestrus

Some strategies to consider for assessing prevalence of anestrus at herd level are listed here.

1) *Performing body condition scoring* (*BCS*). Assessing BCS of cows will provide an indirect indication of whether cows are meeting their energy needs adequately. Performing BCS of the whole herd, paying particular attention to close-up dry cows, fresh cows, and cows in the breeding group, would be of great value. Close-up dry cows that are in high body condition (BCS >3.5/5) should be considered high risk for metabolic disorders and anestrus. A disproportionately large number of cows beyond 60 days in milk with poor body condition (BCS <3.0/5) should also be considered a problem as the prevalence of anestrus will be higher in those herds. Herdsmen should be educated on the importance of BCS, properly trained in the technique, and encouraged to perform condition scoring every two weeks, or at least monthly, and keep a good record of the scores. Inseminating cows in poor body condition must be avoided and breeding delayed until a threshold BCS is attained, as low BCS cows have low conception risk and a high risk of pregnancy loss. Recent studies [74–76] indicate that an acute loss in body condition in the weeks between calving and breeding time in dairy cows has a greater detrimental effect on fertility than the absolute BCS. This finding further emphasizes the need for systematic recording of BCS at set intervals.
2) *Performing gait* (*locomotion*) *scoring*. In herds where gait scoring is not performed, this should be encouraged, routinely, to identify cows that are showing early signs of lameness. Periodic gait scoring, regular hoof trimming, and the use of foot baths must be encouraged to keep lameness in check. Lame cows will often be the last ones to reach the feed bunks and they are less motivated to leave the comfort of their stall to access feed frequently. As a result, they consume less feed, predisposing them to an increased risk of negative energy balance and anestrus. Grouping cows with even early signs of lameness and feeding them separately would be one strategy to improve dry matter intake in these cows, mitigating negative energy balance.
3) *Improving the efficiency of estrus detection*. The use of tail chalk or tail paint to improve estrus detection efficiency is one of the simplest strategies that could be implemented in herds of any size. Using three different colors to mark fresh cows (to determine cyclicity), cows at the end of the elective waiting period (to be flagged for insemination), and previously inseminated cows (for return service) would provide an efficient yet simple system for systematically detecting cows in estrus. In the breeding group, tail chalk scores should be recorded daily for this system to be effective and fresh chalk applied at least once weekly. Applying tail chalk or tail paint on fresh cows 30–40 days before the end of

the elective waiting period is highly recommended. If tail chalk remains largely intact until the end of the elective waiting period, the cow is probably anestrous. I say probably, because "standing estrus," which is essential for tail chalk to be removed completely, is only exhibited by 50% of lactating dairy cows in estrus [41] at 9–12 weeks after calving.

4) *Using ultrasonography*. Transrectal ultrasonography is an excellent diagnostic tool [97, 98] for the objective assessment of ovaries and must be used wherever practical during routine reproductive examinations. Examining the ovaries by ultrasonography at five to six weeks after calving provides great diagnostic value to identify cows at risk of reduced fertility (P/AI) because a cow that is not cyclic (absence of luteal tissue at ultrasound examination) by five to six weeks is less likely to conceive at first service. Diagnosis of the anestrous condition based on ovarian ultrasonography must ideally involve at least two consecutive examinations performed at an interval of 7–14 days [99]. Although two examinations at a 7- to 14-day interval are preferred, even a single ultrasound examination performed at the initiation of a timed-AI (TAI) protocol (e.g. Ovsynch) not only identifies anestrous cows (no luteal tissue) but also assists with the identification of cows at risk of reduced P/AI (cows with ill-defined CL, such as those at proestrus, estrus, and metestrus phases of their estrous cycle) [100–102].
5) *Monitoring progesterone profiles*. Albeit somewhat expensive, monitoring milk progesterone concentrations can be a reliable approach for assessing the prevalence of anestrus in dairy herds. During the early postpartum period progesterone concentrations will be very low and will continue to remain so until first ovulation occurs. Progesterone will rise following the first ovulation but may decline after a few days as the first luteal phase is often short-lived. The second and later luteal phases are more likely to be of normal duration. Dairy cows relapsing back into anestrus after the first postpartum ovulation has been documented [41]; regular measurement of progesterone can assist in determining the patterns of cyclicity in dairy herds. The Herd Navigator® system (DeLaval International) has the capability to measure real-time milk progesterone concentrations at the point of milking in both robotic and parlor systems. It has been claimed that Herd Navigator detects consistently above 95% of all estrus events including silent heats and is very useful in the detection of postpartum anestrus, pregnancy, and ovarian cysts [103]. A recent study validated the efficacy of this system for pregnancy diagnosis in dairy cattle and concluded that >95% accuracy is attainable if pregnancy determination occurred ≥41 days after insemination [104]. While the Herd Navigator is a welcome addition to the reproductive management tool-kit, it is an expensive technology, adaptable only to DeLaval milking systems.
6) *Measuring blood metabolites*. Elevated concentrations of NEFA, an indicator of negative energy balance [79, 92, 105], and betahydroxybutyric acid (BHBA), an indicator of subclinical ketosis, particularly during the first week postpartum [30] can assist in the early identification of cows at risk for anestrus. Low concentrations of glucose and IGF-1 in blood during the early postpartum period also increase the risk of delayed onset of cyclicity [105].

While clinical management of individual cases of anestrus has its place, a whole-herd approach is often more beneficial. For a practitioner to focus only on anestrous cows presented for reproductive examinations during herd health visits is not always the best approach. Instead, taking a proactive approach of whole-herd assessment is highly recommended, particularly in herds with a history or suspicion of high prevalence of anestrus.

Walking through the barns without disturbing the cows to assess stall comfort and stall design and how those might influence cow behavior would be an essential component of the exercise. A poorly designed stall, such as one with limited lunge space, in a free-stall barn, may discourage cows from getting up to access feed several times a day because of the discomforts associated with frequent getting up and lying down in a poorly designed stall.

It is equally, if not more, important for a dairy practitioner to be fully aware of reproductive management practices of the herd and to have regular conversations with the person in charge of reproductive management and the herdsmen/technicians performing estrus detection and AI. In herds that have frequent staff turnover, it would be prudent to ensure that standard operating protocols for chores such as estrus detection, synchronization treatments, semen handling, and AI are available, prominently posted, and strictly followed. The veterinarian must know who is in charge of estrus detection and how frequently and for how long estrus detection is performed each day. In herds where electronic estrus detection systems are used, the system must be optimized for the herd to minimize false positives (wasted semen and labor) and false negatives (missed breeding opportunity). Complete reliance on electronic estrus detection systems must be discouraged, especially in herds where the system is newly installed.

Nutritional Intervention

Interactions between nutrition and reproductive processes are complex [92, 106] and it is often difficult to precisely study effects of specific nutrients on ovarian function. Excessive energy intake during the close-up dry period can result in changes in metabolism that may predispose cows to decreased dry matter intake and higher circulating NEFA during the immediate peripartal period [79]; therefore monitoring feed intake in close-up dry cows is also quite important.

Specific nutritional or nutraceutical interventions to reduce the incidence of postpartum anestrus may include feeding diets high in starch [107, 108] or daily oral administration of monopropylene glycol [109] during the postpartum period. Adding starch [110] or oilseeds enriched in long-chain fatty acids such as linoleic or linolenic acid [111] during the prepartum period has also reduced the interval from calving to first ovulation. One study [112] reported that cows that consumed an energy-restricted (80% of calculated requirement) diet during the entire dry period had lower NEFA concentrations and higher dry matter intake during the postpartum period than cows consuming 160% of predicted energy requirements during the dry period. Thus, several options exist for dietary manipulations that could mitigate negative energy balance and reduce anestrus. However, results are variable and research findings may not always translate into practical or economic solutions under commercial herd settings.

Therapeutic Intervention

Hormonal treatment to induce estrus or ovulation is a more tangible approach that is well within the realm of a veterinary practitioner. Specific hormone therapy can be administered to either an individual animal or a group of cattle as required. The commonly used hormones for treatment of anestrus are progesterone, estradiol, and GnRH, although equine chorionic gonadotropin (eCG), porcine LH, and human chorionic gonadotropin (hCG) have also been used. In herds where estrus detection efficiency is poor and undetected estrus (subestrus or silent estrus) is the primary suspected cause of anestrus, the use of prostaglandin (PG)$F_{2\alpha}$ followed by intensive estrus detection could be quite effective [113, 114]. Of course, $PGF_{2\alpha}$ could also be used in combination with most of the above hormones in protocols that induce or synchronize estrus and ovulation.

Progesterone

A short period of exposure to high progesterone during the postpartum period is important for expression of estrus and for the establishment of normal luteal function. Treating anestrous cows with progesterone increased LH pulsatility, estradiol concentrations, and LH receptors in granulosa and theca cells of the preovulatory follicle [115]. Thus intravaginal progesterone devices (IVPD) can be quite effective in treating true anestrus. When progesterone is used alone, a treatment regimen for true anestrous cases would constitute insertion of a progesterone device for seven to nine days, removal of the device, and insemination at detected estrus. The insertion of a progesterone-releasing intravaginal device (PRID) (1.55 g progesterone) for seven days and its removal at the end of that period induced cyclicity in 64 of 90 (71%) treated cows in contrast to only 33 of 83 (40%) untreated controls [99]; cyclicity was confirmed by the presence of a CL at ovarian ultrasonography 11 days after PRID removal. For therapy of anestrus at herd level, a 7-day progesterone treatment followed by $PGF_{2\alpha}$ injection at device removal is often recommended to ensure luteolysis in cows that were "silent ovulators" (subestrus) and in those that were falsely categorized as anestrous due to poor estrus detection efficiency (unobserved estrus).

The two brands of IVPD available in the US market are CIDR® 1380 (CIDR; Zoetis, Florham Park, NJ, USA; 1.38 g progesterone) and PRID-Delta® (Ceva Animal Health, Cambridge, Ontario, Canada; 1.55 g progesterone). While PRID-Delta is approved in Canada specifically for treatment of anestrus in dairy cattle, CIDR is approved in the USA to treat anestrous, synchronize estrus, and resynchronize the return to estrus in lactating dairy cows that failed to conceive to the previous insemination. To my knowledge neither product has milk withdrawal requirements. The use of oral progestagens (e.g. melengestrol acetate) is not discussed in this chapter because it is (i) registered as a product to improve growth rate and feed efficiency and suppress estrus, and (ii) it is not approved for use in any animal other than beef heifers intended for slaughter (Veterinary Drug Directorate, Ottawa, Canada).

Estradiol

Estradiol alone may be used for inducing estrus in anestrous cattle, but it is not recommended because the ovulatory response to estradiol is often variable. Furthermore, unless anestrous cows were previously exposed to progesterone, even if estradiol treatment induces estrus and ovulation, the ensuing luteal phase will usually be of short duration. However, using estradiol

to induce estrus after a seven-day exposure to progesterone has been found effective in both beef and dairy cattle and has been widely used in New Zealand for treating anestrous dairy cattle. For example, the use of a CIDR device for six days, followed by an injection of 1 mg estradiol benzoate 24 hours after CIDR removal resulted in 87% of dairy cows being detected in estrus within seven days of estradiol treatment; 79% of cows detected in estrus ovulated and a P/AI of 42% was attained [115]. Estradiol is available in different forms: estradiol-17β, estradiol benzoate, estradiol cypionate, and estradiol valerate. To my knowledge no injectable estradiol preparation approved for use in cattle is commercially available in the USA. Estradiol cypionate (ECP), previously marketed by Pfizer Animal Health in the USA and Canada for use in cattle, was withdrawn in 2005. Prepackaged estradiol cypionate is still available in the Canadian market through a small pharmaceutical company. Estradiol products may be legally dispensed by a compounding pharmacist under veterinary prescription in Canada. Although US practitioners may still be able to obtain ECP or other estradiol preparations through compounding pharmacies, such use is illegal because it constitutes compounding of an unapproved animal drug and extra-label use for purposes not specifically approved by the Food and Drug Administration [116]. Considering prevailing concerns about the use of estrogens in food animals, it would be prudent to choose alternative products for management of anestrus.

GnRH

Exogenous administration of GnRH induces LH release as early as the first week after calving in dairy cows, although the response is significantly higher by the second week [117] (Figure 34.7). If a dominant LH-responsive follicle is present at the time of GnRH injection, there is a high probability of it ovulating, especially when the follicle has not been exposed to progesterone. In our laboratory, four of five lactating dairy cows given 100 µg GnRH in the second week after calving ovulated [117]. Inducing an ovulation in anestrous cows with no prior exposure to progesterone could result in short luteal phases, increasing the chances for cows to revert back into anestrus. In one study [99], 93 of 108 (86%) anestrous cows ovulated in response to a GnRH injection, but 20% of these cows reverted to anestrus. Thus, although GnRH can be used independently to induce ovulations in anestrous dairy cows, one must recognize that short luteal phases and reversion to anestrus are drawbacks associated with this approach.

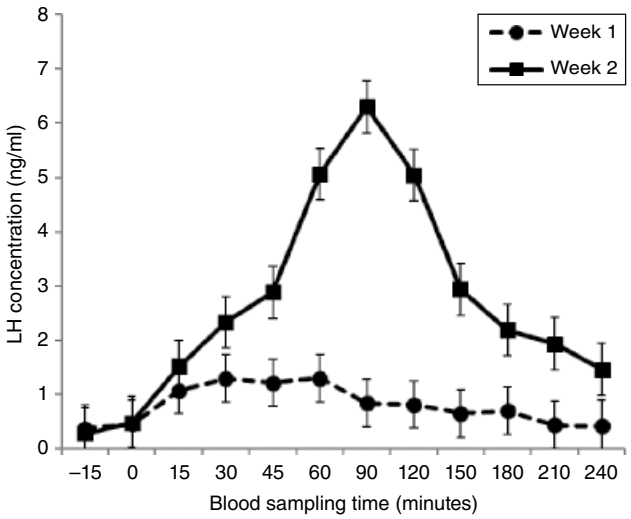


Figure 34.7 GnRH-induced LH release in early postpartum dairy cows when challenged during the first (circles) or second (squares) week postpartum. Cows (n = 10, 5 each week) were given 100 µg gonadorelin acetate intramuscularly (IM) and LH measured in blood samples were collected over four hours. Mean and peak LH concentrations differed ($P < 0.03$) between weeks one and two. A prepartum diet supplemented with oilseeds high in oleic or linoleic acid reduced GnRH-induced LH release in dairy cows during the second week postpartum. *Source:* Redrawn from [117].

GnRH and $PGF_{2\alpha}$ Combination

Using GnRH in combination with $PGF_{2\alpha}$ in a protocol such as Ovsynch [118] followed by TAI (Figure 34.8, A.1) has been effective in the management of anestrous cows. Although the P/AI in anestrous cows following this approach is generally lower than in cyclic controls, several studies [99, 100, 122–125] have reported acceptable P/AI (Table 34.2).

Porcine LH or hCG can be used in lieu of GnRH in the Ovsynch protocol, but these products are significantly more expensive than GnRH. Furthermore, there is limited research on the use of these products in the management of anestrous cattle. In one study [124], replacing the second GnRH injection of the Ovsynch protocol with a commercial preparation of highly purified porcine LH (Lutropin®-V, Bioniche Animal Health, Belleville, Ontario) in predominantly cyclic lactating dairy cows significantly improved P/AI by 14 percentile points (42% vs 28%; $n = 308$). In the same study, when porcine LH was used in lieu of the first GnRH injection of the Ovsynch protocol on a different subset of cows, the ovulatory response to porcine LH treatment was significantly higher than to GnRH treatment (62% vs 44%). Since several studies have reported that cows ovulating in response to the first GnRH treatment of the Ovsynch protocol have higher pregnancy rates after

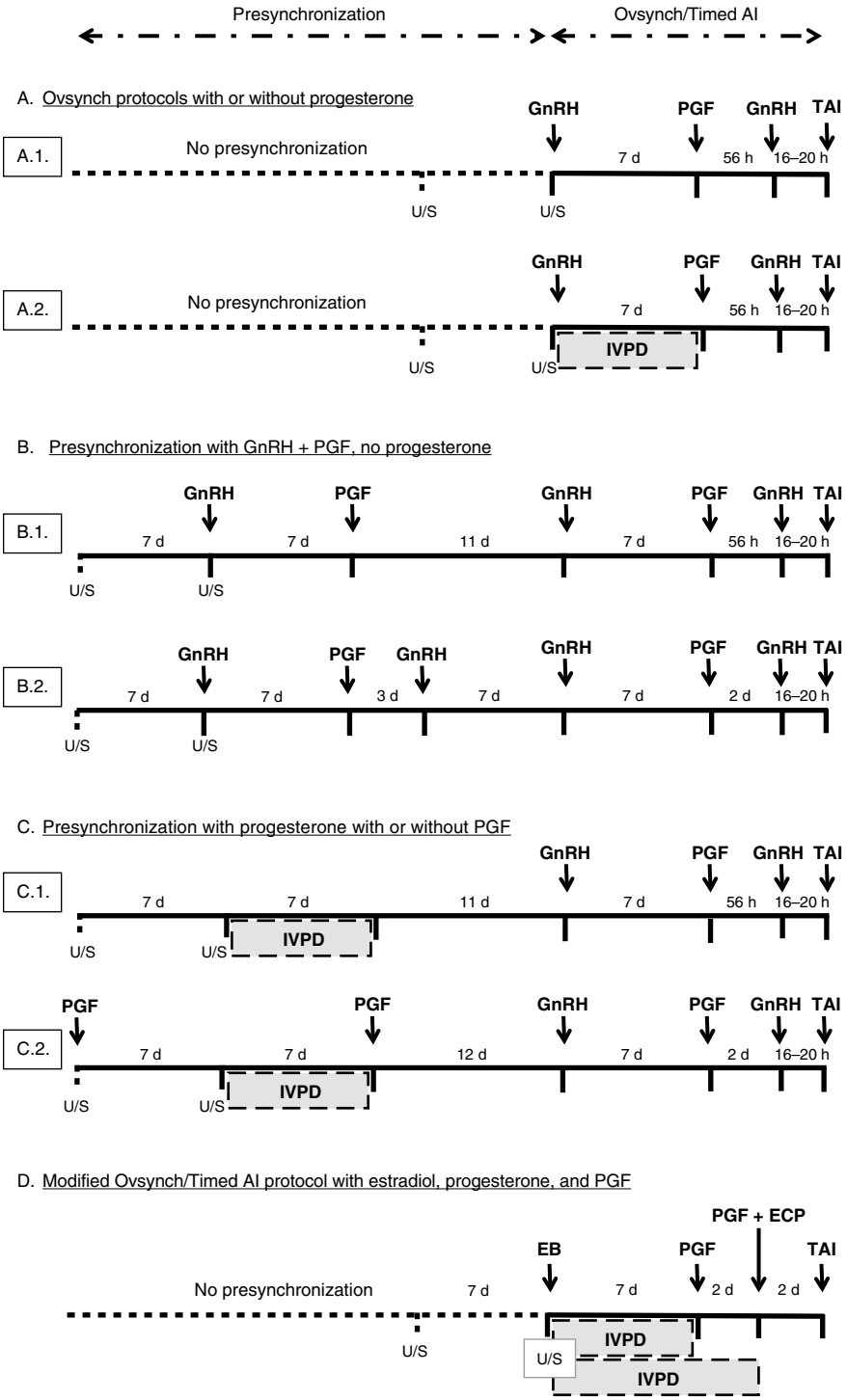


Figure 34.8 Fixed TAI protocols for anestrous dairy cows. A.1 is a standard Ovsynch protocol [118] with no presynchronization or progesterone added. A.2 is a modified Ovsynch protocol that includes an IVPD during the first seven days, with the device inserted at first GnRH and removed at PGFα administration. B.1 and B.2 include presynchronization with either GnRH+PGF (B.1) or GnRH+PGF + GnRH (B.2); in B.1, presynchronizing injections of GnRH and PGF, in that order, are given seven days apart and Ovsynch protocol is started after 11 days [99]. In B.2, two Ovsynch protocols follow back-to-back, with a seven-day interval between end of first and beginning of second protocol (Double-Ovsynch [119]). C.1 involves insertion of an IVPD for 7 days followed by initiation of Ovsynch protocol 11 days later [99]. C.2 [120] is identical to C.1 except that two PGF injections are given 14 days apart, with the IVPD inserted for 7 days starting on the 7th day after initial PGF injection; Ovsynch protocol starts 12 days later. Whether the two injections of PGF given 14 days apart have any value in confirmed anestrus cows was not tested in that report [120]. D [121] is a modified Ovsynch/TAI protocol where 2 mg estradiol benzoate (EB) and 1 mg estradiol cypionate (ECP) replaces GnRH. Two IVPD devices are inserted at EB, with one device kept for 7 days (removed at first PGF) and the second device for 9 days (removed at second PGF/ECP), with TAI occurring 48 hours later. Ovarian ultrasonography (U/S) performed twice, seven days apart, to confirm cyclicity status is important to identify cows that are truly anestrus. Furthermore, confirming ovulation following the first GnRH injection of Ovsynch protocol is highly recommended, where feasible, because anestrous cows that fail to ovulate in response to the first GnRH injection rarely conceive [122].

Table 34.2 P/AI after Ovsynch/TAI in dairy cows classified as anestrous or cyclic.

Percent P/AI			
Anestrous (n)	**Cyclic (n)**	**P**	**Reference**
18.2 (24)	39.0 (303)	<0.02	Moreira et al. [123]
24.1 (116)	38.0 (799)	<0.05	Stevenson et al. [100]
24.4 (45)	30.8 (321)	>0.05	Colazo et al. [124]
30.1 (83)	n/a	n/a	Yilmazbas-Mecitoglu et al. [99]
21.8 (55)	32.0 (259)	>0.05	Colazo et al. [122]
23.1 (26)	32.8 (58)	>0.05	Öztürk et al. [125]

n/a, Not applicable; only anestrous cows were assigned to the study.

TAI [96, 100, 122], using porcine LH in the treatment of individual cases of anestrus has potential value, where such use is legally permissible.

In this regard, P/AI was greatly enhanced in anestrous cows that ovulated in response to the first GnRH treatment of the Ovsynch protocol (46% vs 6%) than in anestrous cows that failed to ovulate [122]. These results strongly indicate that determining the ovulation response to the first GnRH treatment in anestrous cows, by ultrasonography, would have significant value in increasing the probability of pregnancy.

Using a presynchronization treatment (Figure 34.8, B.1, B.2, C.1, and C.2) prior to the initiation of an Ovsynch protocol has a significant advantage in that it creates a physiologically conducive ovarian environment for optimal response to the Ovsynch protocol. Whereas presynchronization with $PGF_{2\alpha}$ alone will perhaps be of little benefit to anestrous cows, using GnRH and $PGF_{2\alpha}$ at strategic intervals prior to the initiation of an Ovsynch protocol can successfully induce cyclicity in anestrous cows [99, 119]. In this regard, a Double-Ovsynch protocol [119] (Figure 34.8, B.2) has been shown to have significant advantages in managing anestrus. At least three reports [119, 126, 127] indicate that the Double-Ovsynch protocol is very effective in inducing ovulation in cows that were non-cyclic at the initiation of the treatment. The Double-Ovsynch protocol does not typically improve P/AI in multiparous cows when compared to a Presynch-Ovsynch protocol, wherein two consecutive injections of $PGF_{2\alpha}$ are given 14 days apart followed by an Ovsynch protocol started 12 days after the second $PGF_{2\alpha}$ injection. Nevertheless, the Double-Ovsynch protocol significantly improves P/AI in primiparous cows, possibly because the incidence of anestrus is higher in primiparous than multiparous cows. All-in-all, a Double-Ovsynch/TAI protocol appears to be an effective strategy to get a greater proportion of anestrous cows pregnant. A recent meta-analysis [128] has evaluated the efficacy of $PGF_{2\alpha}$ minus vs $PGF_{2\alpha}$ plus GnRH-based presynchronization methods prior to the initiation of Ovsynch in lactating dairy cows, comparing 25 publications involving 21 046 cows, and this may be of further interest to the reader.

Progesterone, GnRH, and $PGF_{2\alpha}$ Combination

The incorporation of an IVPD into a synchronized ovulation program such as Ovsynch (Figure 34.8, A.2) is one approach to improve treatment outcomes (ovulation and pregnancy) in anestrous cows [100–102, 122]. An alternative approach is to use an IVPD as a presynchronization treatment (Figure 34.8, C.1, C.2) before the initiation of the Ovsynch protocol. Figure 34.8 presents some of the treatment options for postpartum anestrus in dairy cattle.

Studies show that anestrous cows subjected to Ovsynch plus TAI, with the insertion of an IVPD for the first seven days of the protocol (Fig. 34.8, A.2), have higher P/AI than that of anestrous cows subjected to the same protocol without progesterone (Figure 34.8, A.1). P/AI are comparable to that of cyclic cows subjected to Ovsynch plus TAI with no progesterone treatment (Table 34.3) [100, 122, 129]. A large field trial (12 herds, 1662 cows) conducted in New Zealand on anestrous dairy cows concluded that therapeutic intervention is the most economic option compared with no treatment, and that including an IVPD in the Ovsynch protocol was most cost-effective, attaining the highest net benefit of NZ$80.40 per cow [130]. A meta-analysis of 21 studies (13 762 cows) [131], evaluating the effect of progesterone supplementation within TAI programs concluded that cows lacking a CL (i.e. anestrous) at the initiation of the TAI protocol had greatly improved P/AI when progesterone was given as a single intravaginal insert. Among the cows without CL, progesterone supplementation increased ($P < 0.01$) P/AI at 32 days by 14% (relative risk = 1.14; 95% confidence interval [CI] = 1.04–1.25) and at 60 days by 16% (relative risk = 1.16; 95% CI = 1.06–1.29) compared to untreated controls. Therefore supplemental progesterone is beneficial when provided to anestrous cows and to those without a CL at the initiation of the TAI protocol, whereby P/AI similar to that of cows in diestrus is attainable.

Although stand-alone PGF treatments in a presynchronization protocol are unlikely to benefit anestrous cows, giving PGF before AI in an estradiol–progesterone-based TAI protocol (Figure 34.8D) increased P/AI in anestrous dairy cows [121]. Here, anestrous cows were given a supplementary PGF treatment two days before TAI, secondary to the

Table 34.3 P/AI in anestrous dairy cows subjected to Ovsynch/TAI with or without insertion of an IVPD for the first seven days of the Ovsynch protocol.

Anestrous No IVPD	Anestrous Plus IVPD	Cyclic No IVPD	Cyclic Plus IVPD	Reference
24.1[a] (28/116)	32.3[b] (50/155)	38.0[b] (303/799)	n/a	Stevenson et al. [100]
33.9[a] (139/410)	45.7[b] (178/389)	34.3[a] (142/415)	n/a	McDougall [129]
21.8[a] (12/55)	34.6[b] (18/52)	32.0[b] (83/259)	43.0[b] (104/242)	Colazo et al. [122]

n/a, Not applicable; cows confirmed cyclic did not receive an IVPD in this study.
[a,b] Data with no common superscript within row differ ($P < 0.05$).

luteolytic PGF, to determine if PGF given before AI has a role on fertility independent of its luteolytic effects. P/AI was greater (38.8 vs 13.3%) in normothermic (<39.1 °C) anestrous cows that received the supplementary PGF than in hyperthermic (>39.1 °C) cows. The duration of LH surge, the diameter and volume of the preovulatory follicle, and intrafollicular estradiol concentrations were greater in anestrous cows given the supplementary PGF treatment. In a previous report [132], a low-dose natural PGF (10 mg dinoprost) given concurrently with TAI improved conception rates in lactating dairy cows of undetermined cyclic status. Collectively, these results suggest that the improved fertility in cows due to PGF given before or at AI might be related to changes in gonadotropin release and the endocrine milieu of the preovulatory follicle. Other benefits of PGF treatment may come from a further reduction of progesterone (i.e. in cases of delayed or incomplete luteolysis) at AI which increases the probability of pregnancy [133] and through mechanisms independent of luteolysis, discussed elsewhere by Ambrose et al. [132].

The Ovsynch/TAI protocol has also been used successfully in cows with cystic ovarian follicles [53], although the addition of a progesterone device to this protocol did not improve pregnancy [134]. Ovarian responses documented by ultrasonography and pregnancy rates after TAI in cows with cystic ovarian follicles in the above studies [53, 134] are summarized in Table 34.4.

Table 34.4 Ovarian responses to GnRH treatments, based on ultrasonography, and P/AI after Ovsynch/TAI in cows with cystic ovarian follicles in two studies.

Observation	Reference [53]	Reference [132]
Number of cows	18	109
Mean diameter of cyst	33.9 ± 1.5	33.6 ± 6.0
Ovulation of cystic follicle after first GnRH	0/18 (0)	12/109 (11.0)[a]
Ovulation of an existing follicle after first GnRH	8/18 (44.4)	68/109 (62.4)
New follicle developed after first GnRH	18/18 (100)	102/109 (93.6)
Ovulation of new follicle after second GnRH	15/18 (83.3)	91/109 (83.4)
Cows pregnant 32 days after TAI	7/17 (41.2)[b]	45/109 (41.3)

[a] Some of the smaller cystic follicles (25–30 mm range) ovulated.
[b] Only 17 cows were available for pregnancy diagnosis.

Equine Chorionic Gonadotropin

Studies [135–137] have found that including eCG in a progesterone-based protocol in treatment of anestrous cows is beneficial. Anestrous cows that were subjected to GnRH and six to seven days of an IVPD, followed by $PGF_2\alpha$ plus 400 IU eCG at device removal had increased P/AI at first service (defined as cows that conceived within seven days of their finishing the assigned breeding protocol) by 5.4 percentile points (36.0 vs 30.6%; $P = 0.02$) [135]. Another study [137], in which 1906 anestrous cows from 12 herds were assigned to different ovulation synchronization-based TAI programs with or without an IVPD, found that when eCG treatment was added to a protocol that included an IVPD, P/AI was increased by up to seven percentile units. P/AI and 28-day pregnancy rates were increased ($P < 0.01$) in anestrous cows receiving IVPD + eCG treatment vs those that received neither IVPD nor eCG, if they had passed 60 days postpartum at breeding. Mechanistically, eCG improves the development and ovulation rate of the dominant and

preovulatory follicle(s), consequently improving the quality and function of the ensuing CL, with a positive effect on embryo development and survival, particularly in anoestrus cows [138].

Postbreeding Treatments

Because of the higher risk of pregnancy loss in anestrous dairy cows, postbreeding interventions aimed at improving progesterone concentrations during the early phases of embryonic development have been attempted. Insertion of a PRID for 7 days starting at 4.5 days after AI in anestrous dairy cows tended to reduce pregnancy losses (5.6% vs 33.3%; P = 0.10) between 32 and 60 days of gestation, compared with anestrous cows that did not receive a PRID after AI [122]. In another study, giving a GnRH injection five days after TAI increased P/AI in anestrous dairy cows (45.5 vs 31.1%) compared with cyclic cows [139].

Summary

The underlying reasons for postpartum anestrus in dairy cattle are often multifactorial, but negative energy balance is recognized as a major contributor. To maximize reproductive success, cows that are considered to be at higher risk for anestrus should be identified early and managed for increased dry matter intake. Improving estrus detection efficiency would greatly diminish anestrus due to undetected estrus. Assessment of ovarian status by ultrasonography offers great diagnostic value. Where therapeutic intervention is chosen, a progesterone-based Ovsynch protocol, a progesterone-based presynchronization protocol followed by Ovsynch/TAI, or a Double-Ovsynch/TAI protocol are recommended options. Post-AI treatments with progesterone or GnRH also offer increased P/AI. Treatment of anestrous cows by placing them in a TAI program is the cost-effective alternative to no treatment.

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35

Estrus Detection

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Introduction

Approximately 90% of dairy producers in the USA rely on artificial insemination (AI) to breed their herds [1]. Low estrus detection efficiency is a major cause of poor reproductive performance in dairy farms, which causes substantial economic losses to the cattle industry [2]. Poor estrus detection results in failure to detect and inseminate cows in estrus (inefficiency), insemination of cows at the incorrect time relative to ovulation, or insemination of cows that are not in estrus (inaccuracy). The ability to detect estrus in dairy cows is affected by many factors including human error and inadequate use of heat detection aids, weather conditions, stress, housing, milk production, and endocrine status [2].

Estrus synchronization (Chapter 37) using hormones can help to reduce the time and effort spent on estrus detection and increase its efficiency. Although ovulation synchronization and timed artificial insemination (TAI) protocols (e.g. Ovsynch, Cosynch) have provided dairy producers the opportunity to breed their cows without the need for heat detection, AI based on estrus detection still plays an essential role in the breeding management of most dairy operations [3]. Moreover, a great proportion of dairy cattlemen inseminate their heifers based on estrus detection, and only a small percentage use TAI protocols for the heifers' breeding program. On the other hand, in most beef farms in the USA, breeding programs are based on natural mating. Less than 7% of beef cattle operations use estrus synchronization, heat detection, and AI [4]. In cow-calf beef operations where their genetic progress is based on AI and embryo transfer (ET), estrus detection in combination with TAI protocols represent the cornerstone of the breeding program. In these beef farms, especial consideration should be given to strategies that maximize the identification of animals in estrus. Adequate estrus detection has a significant impact on overall pregnancy rates after a 70-day breeding season, or the number and quality of embryos produced during the ET program.

In general, estrus detection based on visual observation should be performed by observing the herd for one hour in the morning and one hour in the evening. In addition, the appropriate use of estrus detection aids to complement visual observation helps to maintain continuous monitoring of estrus activity with the final purpose of inseminating cows at the correct time relative to ovulation. Some of the estrus detection aids include but are not limited to heat mount detectors or patches, tailhead markers (e.g. paint, chalk, or paste), and teaser bulls, as well as more sophisticated electronic heat detection devices (pedometers, Heat Watch®, etc.). This chapter will discuss the physiological aspects of estrus expression, factors affecting estrus expression and heat detection, and the main management tools used as estrus detection aids, making emphasis on fundamentals, application, advantages, and disadvantages.

Physiology of Estrus

Estrus or heat is a stage of the estrous cycle when the cow is receptive to the bull. It lasts 12–18 hours and occurs as a result of the effects of hormones on the brain behavioral centers. During estrus, the preovulatory follicle grows and secretes high levels of estradiol, which reaches a threshold and induces a positive feedback on the hypothalamus and anterior pituitary, resulting in an increase in luteinizing hormone (LH) concentration and the primary sign of estrus behavior (i.e. the cow stands to be mounted). Estrus behavior coincides with low progesterone levels, high estradiol concentration, and the preovulatory surges of

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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gonadotropin-releasing hormone (GnRH) and LH that result in ovulation 24–32 hours after the beginning of estrus (during metestrus) [5]. This endocrine profile also induces changes in the reproductive tract leading to secondary signs of estrus, including the presence of a clear vaginal discharge, vulvar edema, vaginal hyperemia, and increased uterine tone. During this time, cervical mucus becomes more abundant, clear, and less viscous, which facilitates sperm transport through the cervix. An increased number of leukocytes, particularly neutrophils in the subepithelial layers of the endometrium, can be identified during estrus. These cells are crucial for phagocytosis of bacteria, dead sperm, and cell debris.

Some cows do not show significant signs of estrus but have silent ovulations. This is a condition commonly occurring in dairy cows during the early postpartum period [6, 7]. In contrast, a less common outcome called anovulatory estrus occurs when a cow shows estrus signs but there is a low LH concentration, absence of LH surge, or low responsiveness to the LH surge by the preovulatory follicle [8]. In a field study, the ovulation failure rate in lactating dairy cows detected in heat was reported to be 6.5% [8].

Signs of Estrus in Cattle

In cattle, the most obvious behavioral estrus activity or "primary sign" is standing immobile to be mounted (Figure 35.1). Thus, standing estrus refers to the period when a cow is mounted by other cows and stays quiet without trying to escape. In dairy cows, the number of standing mount episodes during estrus is highly variable (3–140) [9], with an average of 35 mounts of three to seven seconds each. Conversely, some cows do not express signs of standing heat during their estrus, which represents a challenge for the breeding program [10, 11].

A cow in heat also displays secondary signs of estrus (Figure 35.2). These signs may occur before and after heat. She becomes restless and nervous, with increased physical activity [12], raises her tail, and may have a long string of clear mucous discharge hanging from her vulva or on her tail or hindquarters. She has vaginal hyperemia and a reddened, relaxed, and edematous vulva. A roughened tailhead (with fluffed-up hair) or mud on her back or sides indicates that she has been mounted. A cow in heat follows other cows, bellows more frequently, stands beside them, sniffs their vulva, shows Flehmen response, and rests her head on the back of other cows. The cow may behave disorientated, mounting and being mounted but not standing, licking, rubbing, and having aggressive behavior like head butting.

During estrus, an abundant clear vaginal secretion is normal. If the vaginal discharge is bloody, this is also normal but indicates that estrus ended (one to three days ago) and the cow is in "metestrus" and has already ovulated. At this point, it would be generally too late to inseminate this animal, but she should be observed closely for the next estrus in 19 days. If the secretion is cloudy, milk-white, or purulent, the animal may have a



Figure 35.1 (a) Holstein heifer and (b) crossbred Brahman cow displaying primary sign of estrus behavior "standing to be mounted" by a herdmate. *Sources:* Images courtesy of Drs. Roberto Palomares (a) and Antonio Landaeta-Hernandez (b).



Figure 35.2 Holstein cows showing secondary signs of estrus: (a) long string of clear mucous discharge hanging from her vulva and a reddened, relaxed, and edematous vulva; and (b) roughened tailhead indicating that she was mounted. *Sources:* Images courtesy of Drs. Roberto Palomares (a) and Antonio Landaeta-Hernandez (b).

vaginal, cervical, or uterine infection. This condition could reduce the odds of conception.

There may be several cows showing sexual behavior at the same time (especially when synchronization protocols are used), so that it can be difficult to sort out which ones are in heat and which ones may be coming into heat soon. Cows attempting to mount other cows are also part of the so-called sexually active group (SAG). The mounting animals in this group are not necessarily in heat but they may come into heat soon. This behavior results from increasing estradiol concentration (e.g. during proestrus) or social dominance.

An observational study was performed monitoring 200 Holstein–Friesian cows to describe the chronological sequence of estrus behavior in cattle [13]. Visual observation was performed in 3-hour blocks continuously between 04:00 and 24:00 hours and Heatime® neck collars were used as detection aid. The duration of standing to be mounted was 4.7 ± 1.5 hours (range 0.25–18.3 hours). Sniffing the vulva of another cow, chin resting on the rear part of another cow, and being chin rested upon by another cow were the most frequently observed behaviors, with not accepting a mount being the least frequent. The duration between the first and last observed estrus behavior was 14.0 ± 1.9 hours (range 8.5–28.8 hours) [13]. Cows with secondary signs of estrus behavior should be carefully monitored for standing behavior. In dairy cattle, a decrease in feed intake and milk production for no apparent reason may occur during the estrus period. In beef cows, a reduction in milk production during estrus can also be noticed by observing their bawling hungry calves in the pasture.

Factors Affecting Estrus Expression and Detection

The estrus expression intensity and the possibility to detect it can be affected by numerous factors including breed, postpartum period, parity, milk yield, health status, nutrition, weather conditions, stress, hormonal treatment, bull presence, housing, and herd size.

Breed

Estrus behavior including duration (hours) and intensity (mounts per hour) of sexual receptivity is more pronounced in *Bos taurus (8–18 hours)* than *Bos indicus (6–12 hours)*. In addition, a field study showed that cows of dark-colored breeds had greater sexual behavior than white- or red-haired cows [14].

Postpartum Period

The hypothalamus sensitivity to estradiol varies during the postpartum period. Some ovulations occurring during the early postpartum period often occur with mild or no estrus behavior. This is a condition commonly occurring in dairy

cows during the early postpartum period [6, 7]. This might be due to a refractory effect on estradiol caused by high estrogen levels at late pregnancy and parturition. Postpartum anestrus or delayed resumption of ovarian cyclicity is a major factor affecting reproductive performance in beef and high-producing dairy cows [15]. Several studies performed in large commercial dairy farms have demonstrated that approximately 20% (7–41%) of the lactating cows remain in anestrus and anovulation at the end of the 60-day voluntary waiting period (VWP) [16, 17].

Milk Production

High-producing dairy cows had lower estradiol levels and shorter estrus compared with low-producing cows [18]. The higher metabolic rate of estradiol and progesterone observed in high-producing dairy cows has been associated with reduction in duration and intensity of estrus behavior. Nevertheless, reports showed no correlation between estrus expression and level of milk production [19].

Nutrition and Body Condition Score (BCS)

Subnutrition and poor body condition score (BCS) can negatively affect estrus behavior [20]. There may be confounding effects of the diet on the intensity of estrus behavior in lactating Holstein cows. A field study in France demonstrated that there was an increase in the odds of estrus detection based on "mild" secondary signs (instead of standing heat) in cows receiving a high diet (65% corn silage, 5% hay, and 30% concentrate containing soybean, barley, beet pulp, wheat, corn, and minerals) when compared to cows given a low diet (85% grass silage and 15% concentrate). This could be explained by a possible effect of increased milk production in cows consuming high diet decreasing the intensity of estrus behavior [10].

Parity and Lactation Number

There is a contrasting effect of parity on primary and secondary signs of estrus behavior. The length and intensity of the primary estrus sign (standing to be mounted) increases with parity [14]. A multiparous cow expresses "standing estrus" more accurately (during a longer period) than primiparous cows. In contrast, primiparous cows show greater walking and mounting activity during estrus compared to multiparous cows; and this physical activity decreases with each additional lactation [15, 21–23].

Health Status (Lameness and Mastitis)

Although lameness has been associated with delayed resumption of postpartum ovarian cyclicity in cattle, a report showed that follicular development and maturation is similar in lame cows compared with normal animals [24]. Clinical lameness is commonly related to decreased expression of estrus behavior in dairy cows [25]. This reduction in estrus intensity could be due to reduced physical activity and mounting behavior. That is, a lame cow has shorter episodes of standing, mounting, and walking activity during estrus [26]. Alternatively, the reduced estrus behavior in a stressed lame cow might be a consequence of lower progesterone concentrations before estrus and decreased LH secretion, as previously reported [27]. It is also possible that a lame cow stands to be mounted when she is not in estrus since escaping when mounted might increase the pain. Hockett et al. [28] reported that cows with clinical mastitis may have variable reproductive outcomes. They may show normal estrus behavior and ovulation or have decreased LH pulses, lack of LH surge and estrous behavior, low estradiol concentrations, and ovulation failure [28]. In addition, a study showed that subclinical mastitis disturbs the steroidogenic function of small follicles, and these effects are then carried over to preovulatory follicles [29].

Weather and Stress

Several studies have reported significant effects of season on estrus behavior [10, 30, 31]. The effect of heat stress on gonadal steroid production has a significant impact on sexual behavior in cattle. It is well known that the length and intensity of estrus behavior can be dramatically affected by heat stress. A study in beef cows showed that during summer, cows received fewer mounts, with longer intervals between mounts, when compared with winter and spring. Time of ovulation after the onset of estrus was not influenced by the season [32]. Extreme environmental conditions such as heavy rain or high humidity may also lessen or inhibit estrus behavior [10, 30–32]. In addition, there is a variation in estrus activity during the day. Cows increase their mounting and standing behavior during the night and early morning (e.g. from 7 p.m. to 7 a.m.), when the temperature is lower [33].

Housing and Flooring

Duration and intensity of estrus behavior is greater when cattle are kept on dirt compared to concrete, slippery surfaces [34, 35]. Furthermore, the use of rubber mats to cover a concrete floor improved the expression of estrus [36]. In addition, estrus detection is less efficient in cows under tie-stall barn conditions compared with cows observed continuously under free stall barn conditions. Interestingly, barn-housed cattle displayed greater number of mounts during estrus than pasture-managed cattle, which spend a

significant amount of time grazing [37, 38]. It is highly recommended that barn-managed cows be turned out twice daily (morning and afternoon) to be observed during one hour each time.

Presence of a Bull

The presence of a bull has a positive effect on the resumption of post-calving ovarian cyclicity, shortening the interval to first estrus and ovulation via pheromone secretion [39–42]. The presence of the bull may increase the probability to identify cows in heat. However, Landaeta-Hernandez et al. [43] demonstrated that duration of estrus, total mounts received, and intensity of estrus were not affected by the presence of the bull. Moreover, a bull commingling within the herd can inhibit the cow–cow mounting activity [44].

Hormonal Treatments

In general, it is believed that duration of estrus behavior is not different between animals showing prostaglandin (PG) $F_{2\alpha}$ induced estrus versus spontaneous estrus [22]. However, reports showed that duration of natural estrus appears to be longer than estrus induced by $PGF2_{2\alpha}$ (15.3 vs 13.3 hours) [45]. Another study revealed that treatment with progestogens appears to result in higher frequency of heat detection compared to treatment with $PGF_{2\alpha}$ [46]. It is important to emphasize that after hormonal treatments, farm employees are more aware of signs of heat during visual observation.

Population Density

The larger the number of animals in the herd, the greater the sexual interactions among them. When the herd size is increased, the odds of creating cattle SAG is greater. SAG refers to the number of cows in estrus or close to being in estrus interacting and displaying sexual behavior at the same time [47]. A large number of cows in heat at the same time improves the intensity of estrus expression and, therefore, the efficiency of detection [10, 12, 19, 22, 48]. However, excessive number of cows in a small area (overcrowding conditions) might make non-estrous cows stand when they are mounted, resulting in an erroneous heat detection.

Visual Observation as a Method for Estrus Detection

Visual observation is the method most commonly used for estrus detection. The observer's experience, frequency and length of observation, and the time of the day have a significant impact on the estrus detection efficiency.

Heat detection based on visual observation should be performed by observing groups of cows during one hour in the morning and one hour in the evening. Cattle should be observed during their normal activity of social interaction as they move to from housing, feeding, or pasture areas.

Development of observation skills for heat detection requires focus, consistency, and time. The observer must identify the primary sign of estrus (the cow stands to be mounted) without overlooking other secondary signs such as the presence of a clear vaginal discharge, vulvar edema, and increased physical activity. The personnel should be aware also that some cows show short-length estrous signs, while other cows do not show any sign of estrous behavior.

In dairy operations, cows can be observed as they are moved to and from the milking parlor. It is important for the cows to be able to express their sexual behavior to be on dirt, and always avoid observation on slippery concrete floors or floors with standing water. Normally, cattle are unwilling to mount or be mounted when they are on unstable ground surfaces. In beef farms, heat detection often requires observation in the pasture, feeding area, or holding pens. It is critical to not interfere with the cows' social interaction when performing the visual observation. It is common to observe nursing bull calves (approximately five to six months old) following the cows in heat and trying to mount them.

Heat detection efficiency based on visual observation of standing heat varies considerably among herds, from less than 50% to around 90% [49–51]. A significantly high heat detection rate (94%) was reported when visual observation was done twice a day for one hour each, at early morning and late evening. Increasing the observation frequency has a positive impact on the detection efficiency. In a field study using visual observation of both standing and mounting behavior three times per day during 30 minutes each, the heat detection rate improved to 90% [51]. It is highly recommended to encourage the personnel in charge of heat detection by providing a financial incentive (e.g. for each pregnant cow). This has an immediate and direct impact on estrus detection efficiency. Keeping both manual (e.g. notebook) and automated computer-based records of estrus and AI facilitates the anticipation of upcoming heats.

Artificial Insemination Based on Estrus Detection

AI based on heat detection is centered on the AM–PM rule. That is, if a cow shows estrus behavior in the morning, she should be inseminated in the evening. If she shows estrus activity in the evening, she should be inseminated the next morning. Since cows ovulate 24–32 hours after the beginning of estrus, AI performed approximately 8–12 hours

after estrus detection allows sperm to be in the uterus and uterine tubes for enough time to undergo capacitation before being able to fertilize the oocyte. A great proportion of cattle will show estrus behavior overnight. Therefore it is recommended to observe and inseminate cows as late as the light allows in the evening and as early as possible in the morning, especially during the warmest months (July and August). The use of hormonal protocols for estrus–ovulation synchronization permits personnel to combine heat detection plus AI (using the AM–PM rule) and TAI.

In farms where management conditions and heat detection systems allow to identify accurately the beginning of estrus behavior, cows should be inseminated using the AM–PM rule. In contrast, in those farms with low pregnancy rate and poor heat detection, it is recommended to inseminate the cows immediately after they have been detected standing to be mounted [30]. In previous studies an optimal pregnancy rate was achieved when AI was performed from mid-estrus until a few hours after the completion of standing behavior [52, 53].

The efficiency of heat detection affects not only the total number of cows eligible to be inseminated, but also the timing of insemination relative to ovulation and subsequent fertility. For instance, in a previous study, pregnancy rate decreased from 66.2 to 45.4% when AI was performed at 8–18 hours and >30 hours after heat detection, respectively [54]. The timing of AI influences the resulting pregnancy rate by affecting the fertilization rate and embryo quality. A trial demonstrated that performing early AI during standing heat behavior was associated with lower fertilization rates but adequate embryo quality. In contrast, inseminating 24 hours after the onset of estrus resulted in high fertilization rate but low embryo quality. A middle time of AI around 12 hours (6–16 hours) after the onset of estrus (AM–PM rule) resulted in an adequate balance between fertilization rate and embryo quality [55]. This effect was associated with the number of accessory sperm trapped in the zona pellucida. In addition, insemination timing relative to ovulation has an effect on the offspring's gender [54]. The percentage of male offspring is higher with late AI (10 hours before ovulation). In contrast, early AI (20 hours before ovulation) seemed to favor the proportion of female offspring [56].

When AI is based on heat detection, extra caution should be considered for cows previously diagnosed pregnant and showing signs of standing heat. Performing AI in a pregnant animal can cause embryonic mortality or abortion.

Estrus Detection Efficiency

Insemination risk has a great impact on reproductive performance in dairy operations. The number of cows inseminated will depend on the personnel's ability to detect estrus, which can be monitored by multiple parameters of estrus detection efficiency. An essential requisite for assessing estrus detection efficiency is having reliable records of the cows identified in estrus and inseminated (including dates and timing), their pregnancy diagnosis, and calving dates. Farms with severe estrus detection problems have low pregnancy risk, high percentage of repeat breeding, and prolonged intervals from calving to first service and conception. These farms may have a great proportion of cows inseminated when they are not in heat (heat detection inaccuracy), many cows in heat that are not inseminated (heat detection inefficiency), and, commonly, many cows inseminated too early or too late relative to estrus and ovulation.

Ideally, 80–85% of cows should be detected in estrus during the VWP (by 60 days postpartum). Therefore the percentage of anestrous cows should be lower than 15–20%. Farms with adequate estrus detection generally have a calving to first service interval of 71–76 days, most of the cycling cows (>70%) have an inter-estrus interval (IEI) of 18–24 days, and their personnel detect more than 70% of cows in heat.

In farms with inaccurate heat detection, there is a high percentage (>15%) of cows with too short (e.g. less than 17 days) or too long (e.g. 30 days) IEI, many cows are diagnosed pregnant to a service earlier than the last recorded, and a high percentage of cows calve before the calving due date. Inaccuracy of heat detection can be confirmed by determining the progesterone concentrations in milk in a representative number of cows (n = 20) on the day of AI. In these farms with inaccurate heat detection, more than 5% of the samples will have high progesterone concentration (>1 ng/ml), indicating the animals were not in estrus when they were inseminated. In farms with inefficient heat detection, cycling cows are routinely missed before the first insemination, so that the calving to first service interval is prolonged (e.g. 90 days). Furthermore, a high proportion of cows (>15–20%) have an IEI two or three times longer than the expected average of 21 days for cycling cows (e.g. 36–44 or 54–66 days). Moreover, in this case scenerio, the pregnancy risk at diagnosis is lower than 85%, indicating that many estrous cows have been missed between the AI day and the day of pregnancy diagnosis.

Several equations have been created to calculate the estrus detection efficiency (reviewed by Roelofs et al. [30]). These parameters are used to determine the proportion of cows detected in estrus relative to the number of cycling cows during a certain period of time. Some of these methods include percentage of heats detected during and after the VWP and heat detection index, which is calculated utilizing the number of cows detected in estrus, the IEI, the number of days to first service, and the VWP, as determin-

ing endpoints. Another approach includes calculation of the ratio between cows with normal IEI (18–24 days) and cows with prolonged IEI (36–48 days), which should be 4 : 1. Currently, numerous cattle management computer softwares are commercially available and provide a complete evaluation of the reproductive performance, including estrus detection indexes based on the mentioned variables. The information reported by the software will depend on the data collected and recorded from the herd by the personnel, so that continuous monitoring, data collection, and software updates are crucial for accurate reports.

Estrus Detection Aids

Several technologies have been developed for producers to improve estrus detection efficiency by providing continuous monitoring of behavior. These heat detection aids do not substitute for visual observation but have been designed to complement it. The following are the most common estrus detection aids currently used in cattle production.

Rump-Mounted Alert Patches

These include pressure-sensitive heatmount detectors (e.g. Kamar®) which are glued onto the tailhead to assist in identifying females showing standing estrus behavior (Figure 35.3). These patches usually contain a small vial of fluid with a smaller vial of color that breaks when pressure is applied by mounting activity. Animals with an activated (e.g. red color) heatmount detector are considered to be in estrus. In addition, there are self-adhesive heat detection patches with a silver scratch-off surface (e.g. Estrotect®). When the animal standing is mounted, the bright signal layer underneath is shown for easy identification.

Tailhead Markings

Marking the cow's tailhead with chalk, color wax, crayon, or paint (Figure 35.4) helps to identify animals that were mounted. In this case, the paint is rubbed off or smeared down the sides of the tailhead onto the rump, indicating that the cow has been ridden by another cow. This method has the advantage of being less expensive than the rump-mounted alert patches.

Chin-Ball Marker

The chin-ball marker (Figure 35.5) comprises a metal ink reservoir with a large ball bearing held by a harness which is placed under the marker animal's chin. As the marker animal mounts and dismounts the cow in heat, a characteristic mark is left on the cow's back and rump. The paint reservoir usually holds enough paint for about 25 cows, depending on mating behavior. Usually chin-ball markers are used with teaser bulls or testosterone-treated cows.

Teaser Animals

Teaser animals refer to animals that can identify and mount a cow in standing heat but cannot naturally breed her. A "teaser" bull is a bull that has been surgically altered, so that he cannot perform intromission during the mounting activity (Chapter 21). Several techniques can be utilized for this purpose including vasectomy, caudal epididymectomy, penile deviation, and penectomy. In addition, androgenized cows can be used as teaser animals.

A combination of these techniques can be used, such as vasectomy plus penile deviation. An additional benefit of using teaser bulls in beef herds is that their presence stimulates the resumption of cows' postpartum cyclicity, reducing the calving to first estrus interval. One of the disadvantages of using teaser bulls is that they can have a preference for specific cows and miss other cows that are in heat. In addition, some teaser bulls will lose their libido over time once they notice they are not able to breed the cows when they mount them. It is also important to pick teaser bulls that have a docile temperament, as these bulls are separated from the cows before AI.

Vasectomy

Vasectomy consists of removing a section of the ductus deferens from the spermatic cord. The testes and penis continue functioning normally, but transport of spermatozoa to the urethra is interrupted. The sexual activity of the bull remains unaltered and he will detect cows in heat as well as an intact bull but he will not be able to impregnate them. As this method does not prevent penetration, it does not control transmission of venereal diseases in the herd.

Caudal Epididymectomy

This surgical technique involves resection of the epididymis tail (Figure 35.6) to prevent sperm from reaching the ductus deferens and ejaculation. Similar to vasectomy, the disadvantage of this procedure is that the bull continues to penetrate cows and can transmit venereal infections from one cow to another.

Penile Deviation

Penile deviation is commonly done using a surgical technique that translocates the penis and sheath from their natural position to the fold of the flank. This method allows normal erection but not vaginal intromission (Figure 35.7). An alternative technique consists in the resection of a portion (5 cm) of the dorsal ligament of the penis. With this

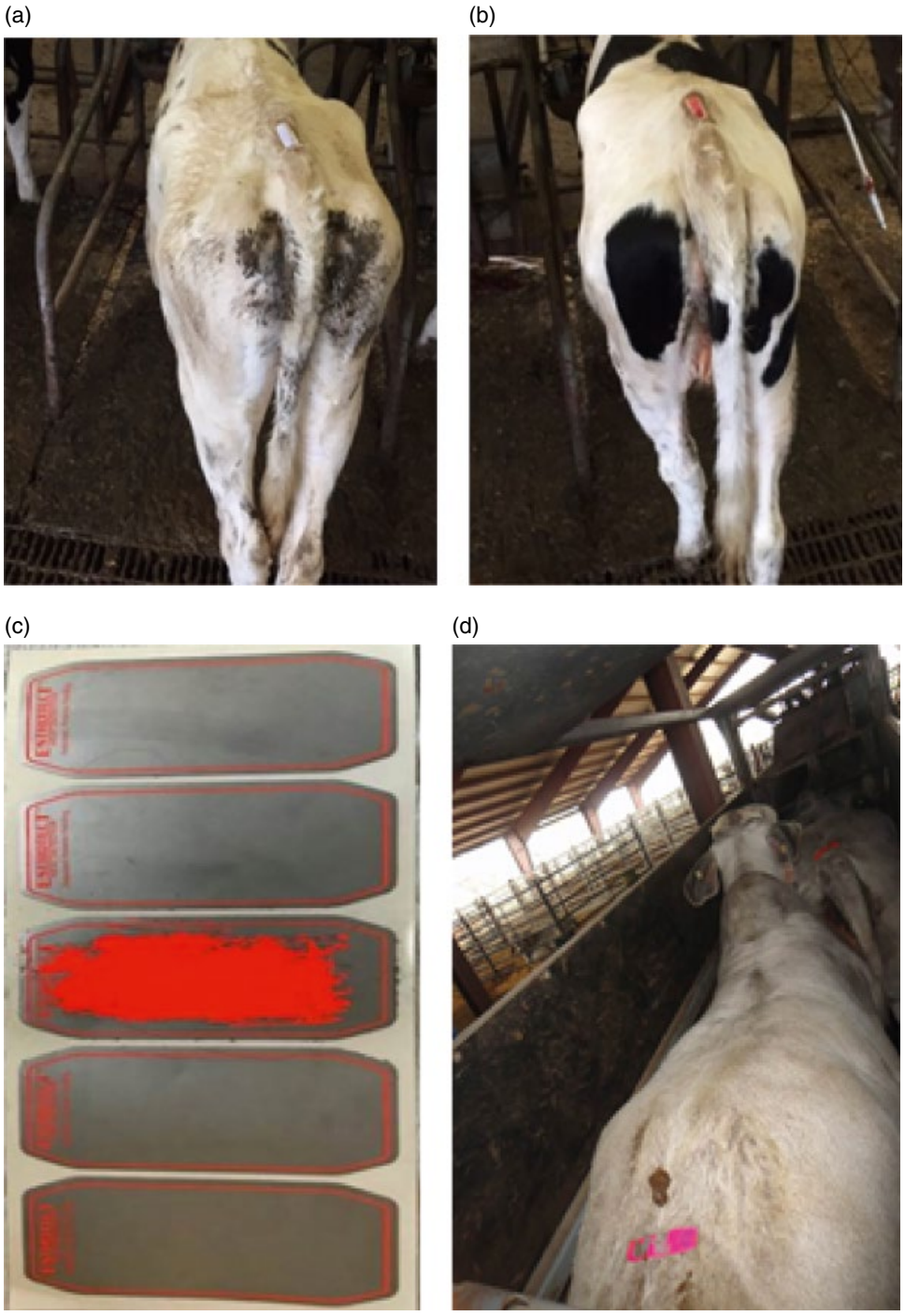


Figure 35.3 Rump-mounted alert patches. Holstein heifers showing pressure-sensitive heatmount detectors (Kamar®) glued onto the tailhead to assist in identifying females showing standing estrus behavior. (a) The heatmount detector is not activated, indicating that this heifer has not been mounted. (b) Heifer showing an activated (red color) heatmount detector, which indicates that she was mounted. (c) Estrotec® heat detector patches intact and scratched. (d) Brahman cow with a patch showing the bright signal layer underneath. *Sources:* Images courtesy of Drs. Roberto Palomares (a–c) and Juan C. Gutierrez-Añez (d).

technique the penis is deviated ventrally during the erection, preventing vaginal penetration. This surgery is faster and simpler than lateral penile translocation and results in a lower risk of postoperative complications compared to penile translocation. However, this technique has not been fully accepted by practitioners since incomplete resection of the penile dorsal ligament results in partial deviation of the penis and vaginal intromission. The advantage of these techniques is that the possibility of transmission of venereal diseases is usually very low unless the bull becomes



Figure 35.4 (a) and (b) All-weather Paintstik® livestock marker can be used to place a thick layer of crayon on top of the tailhead of the cow for detection of mounting behavior. *Source:* Images courtesy of Dr. Roberto Palomares.



Figure 35.5 Teaser bull wearing a chin-ball.

skilled and discovers a way to breed the cow. The main disadvantage is that the bulls can become frustrated and lose their libido.

Penectomy

This technique consists of removing two-thirds of the penis and exteriorizing the remaining end, suturing it to the tissues between the anus and the scrotum. The disadvantage of this procedure is that libido is usually reduced; however, it helps in preventing venereal infections. Ethical issues and animal welfare concerns about this technique might exist and should be considered before using it.



Figure 35.6 Caudal epididymectomy.

Androgenized Animals

This method is based on extra-label systematic injections of testosterone (200 mg intramuscularly (IM) every other day for three weeks) or implant (testosterone plus estradiol) to a cull open cow (not intended for breeding) or freemartin heifer or steer to maintain consistent aggressive sexual behavior. Cows injected with testosterone propionate are usually sexually active, mounting other cows in heat within a few days after treatment. These animals can be equipped with a chin-ball to enhance their efficacy. The benefits of this method are that the cost is relatively low, the androgenized animals cannot spread venereal diseases, and they can be sold for harvest later after the breeding season. It is crucial that these animals be free of feet and leg problems



Figure 35.7 Holstein bull surgically altered showing lateral deviation of the penis during erection. Vaginal penetration is not possible. *Source:* Image courtesy of Dr. Maarten Drost (VISGAR).

(e.g. lameness), have a large body frame, and are dominant, to actively display sexual behavior mounting cows in estrus.

Electronic Heat-Detection Systems

In general, dairy farms have tended to increase their herd size and production and intensified their management during recent years. However, labor has become less available, affecting negatively the efficiency of heat detection. Electronic technologies have been developed to enhance heat detection in dairy cattle by providing continuous monitoring of behavior using tri-axial accelerometers and wireless telemetry. These technologies include electronic rump-mounted devices, and pedometry and radiotelemetric devices that monitor the number of steps and mounting activity. Other electronic equipment that consider additional measures (e.g. milk and skin temperature, electronic noses, rumination) have been developed for this purpose, but are currently under investigation and their efficiency and applicability are still being tested.

Automated Activity Monitoring System (AAMS)

In cattle, increased physical activity is considered a secondary sign of heat. The increase in physical activity in cows has been reported as high as 400% in winter and 175% during summer [57]. Automated activity monitoring systems (AAMS) can continuously monitor physical activity in cattle and register fluctuations in their motion (e.g. head movements). This technology has been implemented with great acceptance in the USA and Europe in the last few years in an attempt to help reduce the labor of visual observation and maintain a continued surveillance.

The system utilizes tri-axial accelerometers in a tag attached to the animal (e.g. a neck collar or ear tag) that constantly determine acceleration forces related to gravity to assess specific variation in physical activity associated with estrus behavior (Figure 35.8). Data are recorded for certain periods of time (e.g. two hours) and transmitted to a transceiver unit (generally at the milking parlor). This device transfers the information to a software installed on an on-farm computer. The individual cow's activity is graphed and, using a mathematical algorithm, an activity index is calculated by the software indicating the variation from average activity in the same time period during a longer time frame (e.g. the last seven days).

Three studies developed at commercial dairy farms using AAMS in cows managed in a free stall barn revealed that approximately 70% of the cows were identified as having increased physical activity [58–60]. In addition, 95% of cows documented to be in estrus by the system ovulated. Further, 35% of the cows not detected in heat by the system ovulated. The results of these studies suggest that AAMS were efficient in detecting cows in estrus but did not detect increased activity in anestrous cows or cows having silent ovulations without heat [58–60]. Similarly, LeRoy et al. [61] performed a study to determine the capability of AAMS to detect estrus for first AI, the accuracy of detection, and the optimum interval from the estrus alert to insemination. Activity monitors identified over 80% of animals for insemination between 55 and 80 days in milk (DIM), but intervention such as TAI would nevertheless be required for the remaining undetected cows. This study suggested that primiparous cows should receive AI 0–8 hours after being detected in estrus by AAMS to optimize pregnancy risk. In contrast, no difference was found in pregnancy risk for the multiparous cows in which AI was performed up to 24 hours after the AAMS alert [61].

A trial compared different AAMS in evaluating steps, motion, neck activity, lying behavior, feeding, rumination, and rumen and vaginal temperature for estrus detection. The study considered visual observation four times a day for heat detection and temporal progesterone profile as a reference standard. Only 56% of cows that ovulated were observed standing by visual observation. The evaluated devices were capable of detecting estrus at least as efficiently as visual observation. The devices were able to detect 15–35% more cows in heat than visual observation [62]. This study recapitulated previous results, indicating that activity patterns increased on the day of estrus. Further, this study and others [63, 64] indicated that decrease in rumination and eating time might also be considered as predictors of estrus in dairy cows [62]. Likewise, another trial using a 3D-accelerometer integrated into an ear tag (SMARTBOW®) for the detection of cows in estrus

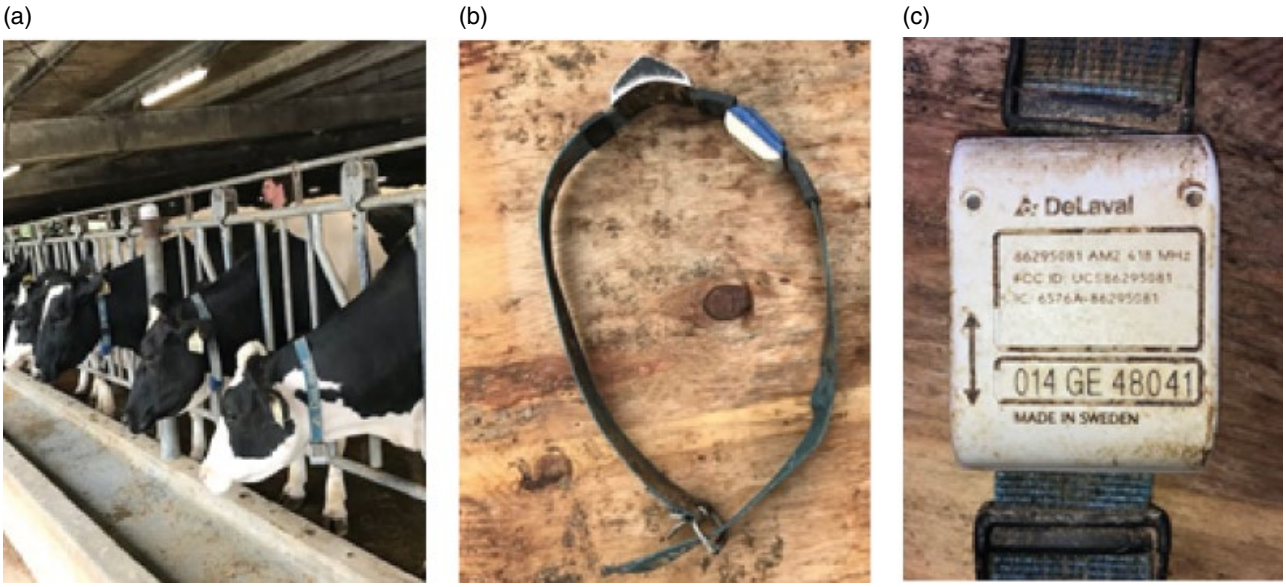


Figure 35.8 (a–c) Automated activity monitoring system (AAMS). Holstein cows wearing activity meters (DelPro™, DeLaval®) attached to neck collars which determine specific variation in physical activity associated with estrus behavior. *Source:* Image courtesy of Dr. Roberto Palomares.

(gold standard: estrus that resulted in pregnancy after AI) demonstrated that the system was appropriate for automated heat detection of multiparous, indoor-housed dairy cows, showing values of sensitivity, specificity, positive and negative predictive values, accuracy, and error rate of 97, 98, 96, 94, 96, and 2%, respectively [65].

AAMS can also determine the duration of the increased physical activity during estrus, which has been reported to be 16.1 ± 4.7 hours (range 4.0–28.0) [58]. These values are similar to the estrus length reported for cows monitored for signs of standing to be mounted (13.4 hours). However, it is important to highlight that secondary signs of estrus behavior increase significantly a few hours (~2 hours) before the onset of standing estrus [59]. Another aspect to consider when using AAMS is that the confined housing conditions in most dairy operations are not appropriate for cows to exhibit full estrus activity, limiting the behavioral indicators used by producers to detect cows in heat. Changes in patterns of lying and standing times at estrus would be a potential parameter for estrus detection in cows within tie-stalls [66]. The evaluation of automated devices using accelerometers revealed an overall increase in total daily standing time during estrous days and longest lying periods on the day after estrus [66, 67]. These studies have also shown that there is a great variation in standing–lying patterns among cows [66, 67].

An eventual goal of these systems is to determine the timing of AI relative to behavioral estrus and ovulation in order to improve overall pregnancy rates. Studies using AAMS revealed that the mean interval from AI to ovulation when inseminating based on the increased physical activity was 7.9 hours [58], which apparently provides enough time for sperm capacitation before fertilization [68]. Another study using AAMS in lactating Holstein cows showed that animals with higher estrous expression had longer intervals from activity alert to ovulation, less ovulation failure, and greater pregnancy rate [69]. Unfortunately, there is a great variation in the interval from onset of activity (and subsequent AI) to ovulation among cows [58]. This variability has a negative impact on pregnancy outcomes. Therefore, in order to minimize the negative effects of this variation, AAMS should be programmed to generate two lists (one list every 8–12 hours) of cows to be inseminated. Another limitation of AAMS is related to the presence of anestrus–anovulatory cows (~20% of the cows in a herd) which fail to be detected with increased activity and are submitted to an ovulation synchronization protocol (e.g. Ovsynch), resulting in poor pregnancy per timed artificial insemination (P/TAI).

The use of AAMS to detect cows for AI in combination with ovulation synchronization and TAI protocols represents a current challenge for herd managers and is becoming a promising tool for systematic AI programs in dairy operations. A study to evaluate the reproductive efficiency of lactating dairy cows managed for first service using a Presynch-Ovsynch TAI protocol with or without detection of estrus using an AAMS reported no improvement in overall pregnancy risk when the presynchronized cows were inseminated based on increased activity and not included for the TAI protocol [59]. A study comparing pregnancy

outcomes in dairy cows inseminated based on AAMS with hormone intervention versus cows inseminated using only a TAI protocol showed no significant differences in pregnancy outcomes between these two management strategies, indicating that AI in cows using AAMS can achieve a level of reproductive performance comparable to TAI [70]. Veronese et al. [71] reported that the use of AAMS for reproductive management of dairy heifers was not advantageous compared with Kamar and did not result in significant improvement in reproductive parameters. The implementation of these systems by herd managers may be affected by hesitancy about their benefits, being unfamiliar with available technology, the excessive information without clear relevance for management [72], the high initial investment, future technology improvement, and availability of future technical support [73].

Pedometers

The number of steps per hour by a cow in estrus is approximately two- to four-fold greater compared with a cow in diestrus [74]. Pedometers are electronic devices that are attached to a cow's leg to count her steps, as an indicator of her physical activity (Figure 35.9). The resulting step count is compared with her baseline count when she is not in estrus. The cow is considered in estrus when her count exceeds the baseline values. When this happens the system sends a signal to the receiver, reporting the event. The efficacy of pedometers is variable (49–90%), since it depends on the housing conditions [75]. Pedometers are more efficient detecting cows in estrus when animals are housed in free stall barns and their benefit is limited in tie stall-housed cows, where they are not able to display increased walking behavior. In farms where animals are moved excessively these systems can provide inaccurate and unreliable results.



Figure 35.9 A cow wearing a pedometer attached to her right rear leg to count her steps, as an indicator of her physical activity. *Source:* Image courtesy of Dr. Antonio Landaeta-Hernandez.

HeatWatch

The HeatWatch® system is an electronic radiotelemetric, pressure-sensitive device that consists of a sensor-transmitter fixed in a cloth tag that is attached to the cow's tailhead region which determines the pressure applied by a mounting cow (Figure 35.10). The transmitter sends a signal to a receiver wired into a computer where the data are stored and analyzed. The transmitters are coded so that each mounted animal sends a unique animal-identifying signal to the computer. The system reports the animal's identification, date, time, and duration of the mounting activity, and which cow is ready to be bred. The HeatWatch® system is very helpful when visual observation for heat detection is inefficient. A disadvantage of this technology is that it has high investment costs and there can be some problems when cows are located under sheds or trees or if there are hills which may inhibit signal reception.

Body and Milk Temperature

During estrus there is a noticeable increase in body and rectal temperature [76]. This variation might be associated with greater physical activity during estrus [30]. However, the specific physiological mechanism involved in the temperature change during the cow's estrous cycle remains unknown.

Thermal infrared (IR) scanning of the cow's posterior body surfaces (gluteal region, vulvar, perineal, and inguinal areas) has been used to detect temperature changes related to ovulation. A trial reported differences of 0.2 °C. The ovulation detection sensitivity was 80%, with high false positive and negative rates [77], not being recommended as an accurate method for routine heat detection. A study in dairy cows using IR thermography and behavioral biometrics showed that variations in radiated temperature from the vulva, muzzle, eye, neck, cheek, and withers as well as tail movements before ovulation were associated with estrus indicators in tie stall-housed cows. The authors stated that this could be used as non-invasive estrus alerts in multiparous cows housed in a tie stall system [78].

Milk temperature may also increase (e.g. 0.3–0.4 °C) on the day of behavioral estrus and has been shown to be a potential alert for estrus detection.



Figure 35.10 (a) and (b) Brahman cows wearing HeatWatch patches (blue and orange) over the tailhead. These cows are in a dry-lot area so that there is close proximity to the receiver for the electronic transmitter. *Source:* Images courtesy of Juan C. Gutierrez-Añez.

Since there is so much variation among animals and body fluids' temperature can be affected by several confounding factors (e.g. season, stress, disease), these methods are not currently considered accurate and reliable for estrus detection.

Vaginal Mucus Electrical Resistance

It is well known that vaginal mucus viscosity and electrical resistance decrease during proestrus and estrus [79]. Previous studies using a conductivity measuring device showed a high variation in vaginal mucus conductivity among cows, suggesting that it is not an accurate or reliable method for estrus detection. Moreover, the tools initially developed were expensive and labor intensive, because cattle needed to be tested frequently to detect the reduction in electrical resistance. In a study evaluating the vaginal conductivity and temperature in 17 female cattle using a wireless vaginal sensor system, 16 of 17 estruses were correctly detected. The interval from detection to ovulation was similar to that observed with standing estrus, suggesting that estrus can be identified in real-time using this new technology [80].

Electronic Nose

Pheromonal odor secreted from the perineal glands near the vagina is a determinant for the bull to identify cows in heat [81]. Efforts have been made to identify chemical compounds associated with estrus [82]. A study was performed to test automatic differentiation between estrous and non-estrous cows using an integrated measurement system consisting of a breath sample device. The results showed sensitivity and specificity combinations of 0.83 and 0.86, respectively, suggesting a potential for automated detection based on breath sampling [83].

Summary

Estrus expression and its detection are multifactorial traits. Estrus detection efficiency depends on the observer's experience, frequency and length of observation, and the method(s) used as detection aid(s). Selection of the estrus detection aid method(s) depends on herd management, cost, labor, efficacy, and usefulness. Detecting cows in heat and inseminating them at the appropriate time relative to the time of estrus behavior and ovulation is vital to achieve an adequate pregnancy rate. Currently, the integration of visual observation with detection aid methods (especially electronic technology) can help enhance estrus detection efficiency in dairy and beef herds. Regardless of the methods utilized in each farm, it is important to frequently monitor estrus detection operating procedures (and retrain personnel), maintain a systematic data collection (e.g. estrus, AI, and calving dates), consistently transcribe the information to the computerized systems, and obtain "useful" reports about reproductive performance that can be interpreted by the herd manager and the veterinarian.

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36

Artificial Insemination

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Introduction

Artificial insemination (AI), the introduction of sperm into the female reproductive tract by means of an instrument, is the oldest assisted reproductive technology [1]. The use of AI in domestic animal reproduction was originally pioneered for sanitary reasons. However, once frozen semen became readily available, the economic advantages of improved fertility and accelerated genetic progress became evident. Since then, AI has become the method of choice for rapidly spreading preferred animal genetics. Numerous bulls have produced hundreds of thousands of insemination doses and offspring. Furthermore, reduced numbers of sperm per insemination dose without compromising fertility has greatly increased the number of inseminations and offspring from genetically superior sires. Several important developments, including novel sperm diluents, new AI techniques, protocols for synchronizing estrus and ovulation, and the availability of gender-selected semen, have further increased the use and importance of AI as an assisted reproduction technique.

Achieving high fertility with AI requires excellent management of all phases of the AI program; it truly requires a team approach. In that regard, personnel responsible for semen collection, processing, and delivery must all correctly perform their tasks, as any deficit will reduce fertility success. Furthermore, the innate fertility of bulls and cows is of vital importance.

Large commercial AI centers generally have protocols and quality control procedures in place to ensure that good-quality semen is marketed. However, once the semen has been sold, fertility depends on the ability of others to correctly handle and thaw the semen and to correctly inseminate cows at the proper time. The primary objective of semen handling is to conserve fertilizing competency, which is accomplished by minimizing exposure of sperm to deleterious conditions. Damage to sperm during cryopreservation, storage, and thawing has been attributed to cold shock, ice crystal formation, oxidative stress, membrane alteration, cryoprotectant toxicity, and osmotic changes [2]. Knowledge of semen tank management, proper thawing and semen handling techniques, and sanitary insemination in the correct location at the correct time are critical and should be periodically reviewed.

Semen Tank Management

Frozen semen is stored in a specialized tank containing liquid nitrogen. These tanks have a rugged outer jacket (aluminum or stainless steel) and an inner compartment that contains liquid nitrogen. The space between the inner and outer jackets is insulated and under extreme vacuum. The lid is attached to a hard foam cylinder that protrudes into the neck of the tank to insulate liquid nitrogen and frozen semen from outside temperatures, thereby minimizing evaporation. However, the tank is not airtight; the liquid nitrogen releases gas as the temperature fluctuates (if the tank was tightly sealed, it might explode). Recent technical progress in design and construction has resulted in user-friendly good-quality semen tanks, including those with extended holding intervals (6–12 months). It is noteworthy that holding intervals vary according to tank model and the frequency with which it is opened. It is essential that the nitrogen content is routinely monitored and additional nitrogen added as required.

With newer semen tanks, maintenance of very low liquid nitrogen temperatures in the inner chamber is possible due to high-quality solid insulation material and vacuum. Regardless, all tanks are susceptible to damage from mishandling. The inner chamber containing liquid nitrogen is actually suspended from the outer shell by the neck tube.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 36.1 Fluctuating temperatures in the neck of a typical semen storage tank.

Location in neck of storage tank	Temperature (°C)
Top of neck	2.2 to 12.2
2.54 cm from top	−15 to −22.2
5.08 cm from top	−40 to −46
7.62 cm inch from top	−75 to −82
10.16 cm from top	−100 to −120
12.7 cm from top	−140 to −160
15.24 cm from top	−180 to −192

Source: Adapted from [3].

Consequently, any abnormal stress on the neck tube caused by substantial force or an excessive swinging motion can crack the tube, resulting in vacuum loss. Puncture of the outer shell will also lead to vacuum loss. Welding the tank exterior should be avoided, as this could also cause vacuum loss. Since vacuum is the major insulating component of the tank, vacuum loss causes an increase in temperature within the inner chamber and rapid evaporation of nitrogen. Accumulation of heavy frost at the top of the tank indicates rapid evaporation of liquid nitrogen and tank failure. Although semen can be stored at −196 °C indefinitely with minimum liquid nitrogen, a minimum depth of 5 cm should be maintained.

Semen storage tanks should be stored away from direct sunlight in a cool, clean, dry, dust-free, and well-ventilated environment. Tanks should be elevated on a wooden pallet and never stored directly on a concrete floor (to prevent corrosion on the bottom of the tank). Furthermore, tanks should not be stacked on top of each other. The tank should be placed in an area where it can be observed frequently to detect excessive evaporation of liquid nitrogen. The tank should not be laid horizontally and rolled. Storing the tank on a wooden or plastic dolly with wheels facilitates movement of the tank. Tanks should be properly secured during transportation in a vehicle to minimize damage or spillage.

Frozen Semen Storage

Most semen is packaged in 0.25- or 0.5-ml straws (sex-sorted semen is consistently in 0.25-ml straws). The straw is placed in a goblet with four other straws from the same bull. Two goblets are packaged onto a metal cane that has the bull's code number printed on top. Canes are stored in canisters in a semen tank. Storage and handling of 0.5- and 0.25-ml straws are similar.

Maintenance of low temperatures is the key to successful storage of frozen semen. During cooling and freezing, microenvironments are created within the semen straw. Each chemical component of extended semen freezes or solidifies at a different temperature. Water begins to freeze as temperatures are decreased below 0 °C, forming ice crystals that remain somewhat unstable at temperatures above −80 °C [3]. This instability is thought to be caused by recrystallization of the ice. Also, as water is converted to ice, sperm are exposed to the remaining concentrated solution of salts and other components of the extender which freeze at temperatures considerably below the freezing point of water. Instability of ice and concentrated solutions are harmful to sperm. Dehydration during cryopreservation has an important impact on sperm; dehydration decreases the risk of intracellular ice crystal formation, but excessive dehydration is also detrimental [4]. Adjusting the osmolality of the diluents is critical, as it influences water fluxes during cryopreservation [4]. Fortunately, cryoprotective agents and optimized freezing programs help minimize sperm damage. However, semen must be kept well below critical temperatures where the recrystallization of ice begins to occur (−100 to −80 °C). Temperatures fluctuate dangerously from the low to upper third of the neck of the tank [3] (Table 36.1). Furthermore, stored semen can be exposed to adverse high temperatures when removed from the tank for thawing, when transferring semen from tank to tank, and when handling semen within the neck when trying to locate and thaw a specific straw. In addition, other semen straws in goblets and canisters are also exposed to high temperatures. The thermal response of semen in 0.5-ml straws when raised to 5 cm from the top of the tank and exposed to an environment temperature of 22 °C (ambient room temperature) and 5 °C (refrigerated room temperature) is shown in Table 36.2. The time to reach critical ice recrystallization temperature (−100 to −80 °C) is approximately 10–20 seconds for both temperatures at half or full capacity. Thermal injury to sperm is permanent and cannot be corrected by returning semen to liquid nitrogen. To assure maintenance of sperm viability, canes and canisters should be raised into the neck of the tank and kept below the frost line only for five to eight seconds. If necessary, the canister should be lowered back into the tank for cooling and subsequently brought back to the neck of the tank.

The number of sperm per straw is variable, with some reduction in number common for high-demand elite bulls and substantially lower numbers for sex-sorted semen. Higher dilutions (2 million sperm per 0.25-ml straw) did not affect the proportions of linearly motile spermatozoa, membrane integrity or stability, or chromatin integrity immediately after thawing compared with a low dilution

Table 36.2 Thermal response of semen straw exposed to refrigerated and ambient temperature (temperature observed in the upper portion of the semen storage tank).

		At full capacity[a]								At half capacity[b]							
		At 5 °C				At 22 °C				At 5 °C				At 22 °C			
Tank capacity (l)	Normal working duration (days)	5 s	30 s	60 s	120 s	5 s	30 s	60 s	120 s	5 s	30 s	60 s	120 s	5 s	30 s	60 s	120 s
10	45	−180	−82	−52	−31	−180	−77	−47	−28	−180	−79	−46	−32	−180	−67	−46	−25
20	120	−180	−80	−54	−34	−180	−75	−44	−28	−180	−80	−47	−32	−180	−63	−41	−24
35	123	−180	−85	−56	−36	−180	−73	−42	−25	−180	−85	−44	−28	−180	−60	−37	−22
47.5	76	−180	−90	−58	−37	−180	−70	−40	−22	−180	−85	−42	−28	−180	−58	−35	−18

At 5 °C, the tank was stored in a refrigerated room and measurements were recorded; at 22 °C, the tank was stored in a room at ambient temperature and measurements were recorded.
[a] Full capacity means the semen tank was filled with liquid nitrogen to full capacity (e.g. 10 l of LN_2 in a 10-l tank).
[b] Half capacity means the semen tank was filled with liquid nitrogen to half capacity (e.g. 5 l of LN_2 in a 10-l tank).

rate (15 million sperm per 0.25-ml straw). Further, there was no difference in pregnancy rate between dilution rates [5]. Regardless, it is well known that some bulls require more sperm per insemination dose to maintain fertility. Furthermore, due to very low numbers per insemination dose, the fertility of sex-sorted sperm is almost inevitably decreased.

Tips to Minimize Thermal Injury

- Develop a semen inventory system and mount it on a wall near the tank. Try to keep semen from one bull on each rack. Label canister and goblets with bull number/name. Such systems prevent unnecessary searching and exposure of semen to dangerously high temperatures in the neck of the tank.
- Organize rapid transfer of semen between tanks. Involve two people and arrange the tanks side by side. If possible, fill the tanks with nitrogen before transfer. Raise canisters only to a level necessary to locate the rack of semen to be transferred.
- When preparing to thaw semen, raise the canister into the lower portion of the neck where the specific goblet of semen can be grasped, then lower the canister further into the neck. Secure the goblet as low as possible in the neck, thus protecting other straws from thermal injury. If straws cannot be easily removed, bend the top tab of the goblet (45° angle) to improve access.
- Use forceps to remove the straw and to transfer it to the thaw bath. As soon as the straw is retrieved, quickly lower the rack of semen and canister into the body of the tank.

Semen Thawing Method

Various methods for thawing semen in straws have been recommended, including a variety of water bath temperatures and thawing periods, shirt pocket thawing, air-thawing, and thawing in the cow. Regardless, it is recommended that all persons on a farm use the same method to thaw semen. The National Association of Animal Breeders recommends a semen straw be immersed in a water bath at 30–35 °C for 40 seconds. The thawing time for straws should be a minimum of 30 seconds [6]. Thawed semen should be inseminated within 15 minutes after thawing and drastic decreases in post-thaw temperature must be avoided [7].

In pocket thaw methods, the initial pocket temperature was 30 ± 1 °C. However, one minute after pocketing, the temperature decreased to approximately 19 ± 1 °C, which could have adverse effects on sperm parameters. Semen in straws began to liquefy approximately three minutes after pocketing, with thawing completed by five minutes [7]. The results of semen viability assays after pocket thaw differed widely among experiments. The rate of thawing in a shirt pocket could be quite variable depending on the ambient temperature and the insulating effect of different types and thicknesses of clothing. Furthermore, the thaw rate was much slower for pocket-thaw compared with a water bath [7]. More rapid thaw rates result in higher post-thaw viability compared with the pocket-thaw method [7]. However, a recent study claimed that irrespective of improved *in vitro;* semen quality with a fast thaw rate, these semen quality measures did not increase *in vivo;* fertility compared with a pocket-thaw method [8]. If frozen semen is prepared to permit flexible thawing, the thaw method used, whether pocket or warm water thaw, should not affect conception under commercial conditions [8]. Although some sire and extender (egg-yolk citrate vs non-heated whole milk extender) combinations seem to be tolerant to thaw procedures, other combinations are more sensitive, resulting in reduced post-thaw sperm survival, conception rates, or both, in response to thaw methods [9].

Concurrently thawing multiple straws of semen can facilitate efficiently breeding a large number of cows without compromising semen quality or conception rates. Despite several reports that more than one straw can be safely thawed, caution must be employed while preparing multiple guns, because it is paramount to follow proper procedures for straw preparation and thermal protection and to always work within skill and time constraints. An experienced inseminator can thaw multiple straws of semen and prepare insemination guns to breed up to four cows within 20 minutes, without an adverse effect on conception [10]. Another study concluded that, on average, conception rates differed between professional AI technicians and herd inseminators. However, elapsed time from initial thaw to completion of fourth AI and sequence of insemination (first, second, third, or fourth) had no effect on conception rate within the inseminator group [11]. Oliveira et al. [12] concluded that sequence of insemination after simultaneous thawing of 10 semen straws can differently affect conception rates at timed AI, depending on the sire used. Regardless of number of straws thawed simultaneously, it is vital that straws are agitated immediately after immersion to prevent straws from clumping together, resulting in a decreased rate of thaw.

Semen Handling After Thawing

Mixing water and semen will cause irreversible sperm injury. Hence, on removal of the straw from the water bath, the straw should be dried (typically with a paper towel)

before it is cut. If the straw is defective (e.g. perforated), it should be discarded.

Prevention of cold shock is critical for post-thaw semen handling. Cold shock inflicts irreversible injury to sperm and is caused by rapid post-thaw decrease in temperature; this occurs when semen is thawed and then subjected to cold environmental temperatures before being inseminated. Cold shock decreases motility and fertilizing competency due to irreversible changes in the sperm plasma membrane. The severity depends on the rate and duration of temperature drop. The most obvious sign of cold shock is loss of motility which is not regained on warming the semen. There is also a decrease in the rate of fructose breakdown by the sperm and a decrease in oxygen uptake and in adenosine triphosphate (ATP) concentrations, which can now be no longer synthesized and used to supply energy. Cold shock caused an influx of sodium (186.2 ± 9.3 vs 140.7 ± 10.2 mg/100 g wet weight) into sperm, as well as an efflux of potassium (101.6 ± 6.8 vs 155.4 ± 9.6 mg/100 g wet weight) and magnesium (8.1 ± 0.2 vs 11.8 ± 0.5 mg/100 g wet weight) from the sperm [13]. The higher total calcium concentrations in bovine seminal plasma than in sperm are primarily due to a much higher concentration of complex calcium in seminal plasma. The concentration of protein-bound calcium was approximately the same in spermatozoa and seminal plasma, whereas concentrations of ionized calcium were lower in seminal plasma than in spermatozoa. Cold shock significantly increased the concentration of total calcium in spermatozoa, with a corresponding decrease of total calcium in the seminal plasma, due to a decreased content of ionized calcium in the seminal plasma and a simultaneously increased content of complex and protein-bound calcium in spermatozoa. In contrast, there was no increase of calcium in spermatozoa following slow cooling.

Cold shock occurs most frequently when breeding is performed in cold weather and especially when a warm water bath is used. The high surface to volume ratio of a straw makes it vulnerable to cold shock. Saacke [14], using the 0.5-ml French straw, measured the effect of static ambient temperatures (21, 4, and 16 °C) on the rate of temperature drop in semen after thawing during preparation of the inseminating rod (Table 36.3). The temperature drop was only 3–6 °C for an ambient temperature of 21 °C; furthermore, warming the rod was effective in countering temperature drop. However, during preparation at 4 and 16 °C ambient temperatures, the drops were 15 and 20 °C, respectively, and preparation of the AI gun for insemination at these two temperatures only postponed the temperature drop. Clearly, precautions against cold shock must be implemented during preparation of inseminating guns in a cool environment.

Tips to Prevent Cold Shock in Cold Weather

- Perform semen thawing and loading of the AI gun in a sheltered heated area located close to the animal to be bred. Then insulate the AI gun by tucking within clothing or in an AI gun warmer and carry it to the breeding chute.
- Perform preparation of the AI gun and breeding in a sheltered heated area.
- Utilize an AI gun warmer. An AI gun warmer is a portable, heated, temperature-controlled carrying case that maintains multiple loaded AI guns at 35 °C.

Table 36.3 Temperature of semen (°C) in 0.5-ml French straws after water bath thaw at 35 °C during preparation of an AI gun at 21, 16, and 4 °C.

	Preparation at 21 °C		Preparation at 16 °C		Preparation at 4 °C	
Stage of preparation[a]	Warmed[b]	Unwarmed[c]	Warmed	Unwarmed	Warmed	Unwarmed
Thaw straw	35	35	35	35	35	35
Dry with paper towel	34.5	34	31.5	29.5	31.5	31
Load in gun	34.5	32	28.5	22	29	24.5
Trim the straw	34.5	31	27.5	18.5	28.5	22.5
Apply sheath	33	29.5	22	15.5	23.5	21.5
Wrap with paper towel	32.75	29.5	19.5	13.5	22	20
Tuck under shirt close to body	32.5	29.5	19	13	21	19.75

Source: Adapted from [14].
[a] Preparation of AI gun from thaw to tuck took approximately one minute.
[b] The AI gun was warmed by rubbing rapidly with the hand (warmed) or was maintained at ambient temperature (unwarmed).

Semen Straw Retrieval, Thawing, and Handling Tips

In order to obtain optimal results, frozen semen should be handled and thawed carefully. Insemination equipment should be kept clean and dry (including straw trimmers, which often become heavily soiled). A thermometer should be routinely utilized to monitor the temperature of a water bath, especially when several straws are thawed concurrently. The thermometer should be periodically checked for accuracy (at least every six months).

On retrieving straw from the semen tank, shake the straw gently to remove excess liquid nitrogen that may be retained in the cotton plug and promptly place in 35 °C water to thaw for at least 45 seconds. The thawing should be timed with a watch. While the semen is thawing, prewarm the barrel by stroking it vigorously with a clean paper towel or by placing it close to your body for several minutes in advance of loading. After thawing is completed, remove the straw from the thaw water and wipe it completely dry with a paper towel, and protect the straw from sunlight and rapid cooling. The straw should be held with the crimp upright and gently tapped so that the small air space is adjacent to the crimp. Place the cotton plug end of the straw into the AI gun. Only clean sharp scissors or a straw cutter should be used to cut the straw, perpendicular to the long axis, approximately below the crimped seal to attain a good seal with the AI gun sheath. Slide the plastic sheath over the straw and gun and firmly attach the sheath and gun together. Inspect the straw end of the gun to ensure a proper seal between sheath and straw. The loaded insemination gun is wrapped in a clean paper towel or in a plastic sheath cover to provide both thermal and hygienic protection and the gun is tucked inside clothing or a gun warmer for transport to the animal. The interval from removing the straw from the tank to AI should not exceed 15 minutes.

Insemination

Higher fertility requires proper insemination methods. Several insemination methods have been developed for AI in cattle: uterine body insemination, bilateral cornual insemination, deep uterine horn insemination, and intrafollicular insemination. The most common approach is to use a rectovaginal technique to deposit semen in the uterine body, immediately anterior to the internal os of the cervix. Uterine body and cornual insemination require skill to pass the insemination rod through the cervix. Various training programs are available. For example, many AI studs offer intensive three to five day training schools or provide instructions for individuals on the farm. Most agricultural colleges devote a whole course or part of a course to the technique of AI. However, the intensity of training and specific recommendations given to the participants varies considerably among training programs.

Developing the skill to pass the insemination rod through the cervix should not be the only objective of an AI training program. Along with mastering cervical penetration, the importance of sanitation must be emphasized and skills perfected to consistently identify the proper site of semen deposition and accurately deposit the semen. In addition, trainees should obtain a working knowledge of reproductive anatomy and appreciate the essentials of a sound reproductive management program. Failure to understand the anatomical and functional relationships of various tissues and organs of a cow's reproductive system may lead to insemination errors.

The loaded AI gun is placed in a sanitary plastic cover for protection from vaginal contamination (Figure 36.1). The cow should be adequately restrained, with care to minimize stress (as increased blood cortisol concentrations will supress a luteinizing hormone surge). The palpating arm is introduced into the rectum and the cervix located and firmly grasped (Figure 36.2). The vulva is cleaned with a paper towel, the covered AI gun is inserted into the vagina without contacting the lips of the vulva, and the AI gun is passed through the vagina to the external cervical os (Figure 36.3). Care should be taken to avoid the urethral orifice, vaginal fold, and vaginal fornix. To avoid the possibility of entering the urethral opening (on the floor of the vagina), insert the inseminating rod into the vulva at an angle of 30–40°. The fornix of the vagina tends to stretch rather easily when the AI gun is pushed forward and beyond the cervix. This may give the false impression that progress is being made in advancing the rod through the cervix when really it is above, below, or to either side of the cervix (Figure 36.4). However, one should be able to feel the rod within the vaginal fold (Figure 36.5). When the rod is within the cervix, its tip cannot be felt. The sanitary cover is punctured (Figure 36.6) and the instrument is advanced through the cervix into the base of the uterine body. There are approximately three misaligned cervical rings through which the rod must pass; the rod is advanced by manipulating the cervix and keeping consistent firm pressure on

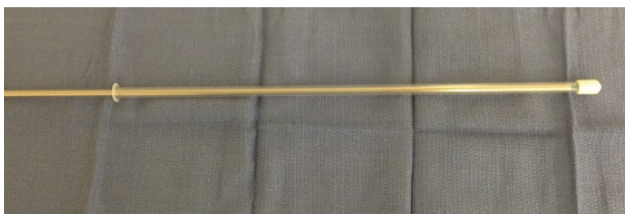


Figure 36.1 AI gun with protective sheath.



Figure 36.2 Locating and grasping the cervix.



Figure 36.3 Locating the external os of the cervix.

the instrument (Figure 36.7). Care should be taken to avoid catching the tip of the rod on cervical folds. The goal for semen deposition is within the uterine body. Since the uterine body is quite small, accurate AI gun placement is paramount. Generally, this site is identified by feeling the internal os of the cervix and the tip of the AI gun as it passes through the internal os and enters the uterine body (Figure 36.8). The tip of the gun should be positioned so that it is approximately 1 cm anterior to the internal os (Figures 36.9 and 36.10); this can be verified with the tip of the index finger of the hand within the rectum. Once the inseminating rod is correctly positioned, the cervix should



Figure 36.4 Avoiding the vaginal fornix.



Figure 36.5 Locating the tip of the AI gun in the anterior vagina.



Figure 36.6 Puncturing the AI gun protective sheath at the external os of the cervix.



Figure 36.7 Passing the AI gun through the cervix.

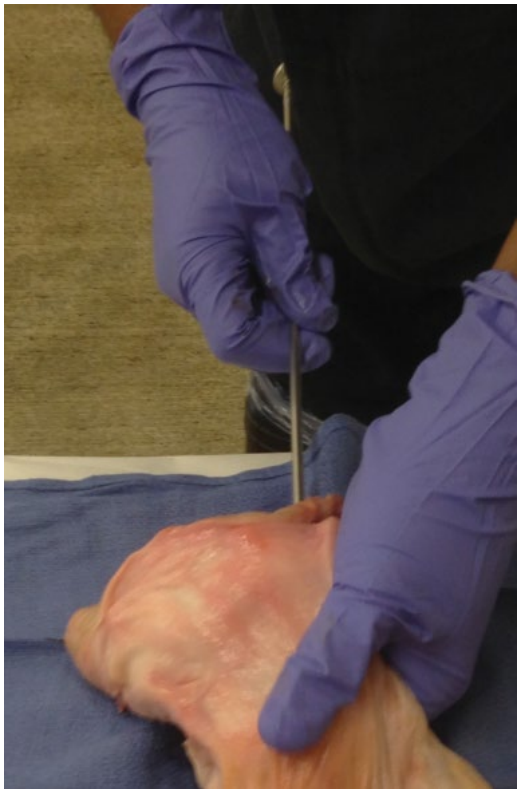


Figure 36.8 Feeling the AI gun in the uterine body.



Figure 36.9 The tip of the AI gun in the uterine body.

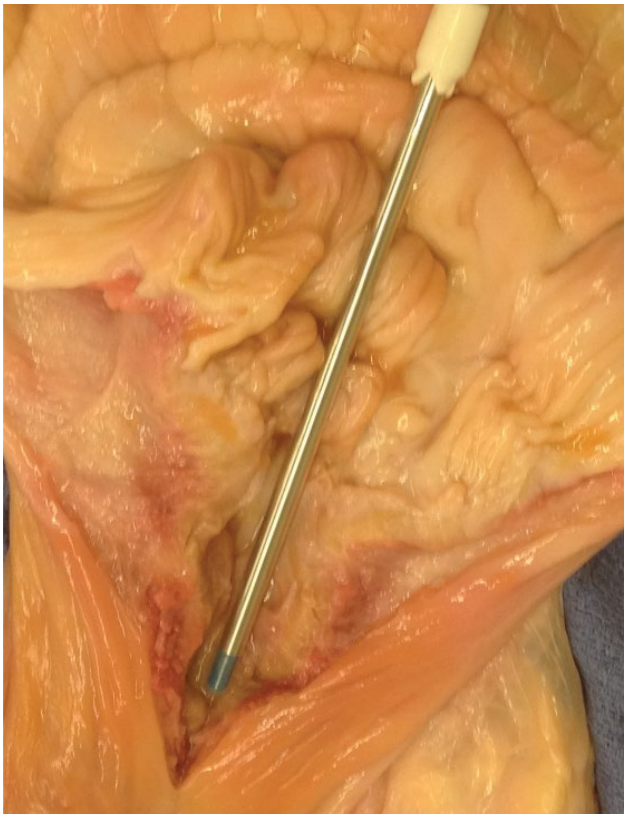


Figure 36.10 Semen deposition site (uterine body).

be held parallel to the long axis of the cow and semen is deposited over a period of approximately five seconds. Slow delivery maximizes the amount of semen delivered from the straw and minimizes the flow of semen unequally into one uterine horn. During the process of semen deposition, one must ensure that the fingers of the palpating hand are not inadvertently blocking a uterine horn or misdirecting the flow of semen in some manner. Care should be taken to avoid pulling the AI gun back through the cervix while depositing the semen. If the cow moves during semen deposition or if the rod slips, reposition the rod to assure proper placement.

Even though the standard recommendation is to deposit semen in the uterine body, bicornual insemination results in no change in fertility [15, 16] or improved fertility [17]. Andersson et al. [18] reported similar pregnancy rates for ipsilateral (to the ovary with the preovulatory follicle) cornual insemination with a lower insemination dose (2 million sperm) compared with uterine body insemination with normal dose (15 million sperm); however, fertility was reduced for lower doses inseminated in the uterine body or bilateral cornual insemination compared with higher dose insemination. It should be noted that anterior cornual insemination did not increase sperm retention rate compared with uterine body insemination. Although factors controlling sperm transport and retention are complex, it is clear that retrograde transport of sperm occurs regardless of site of deposition.

Verberckmoes et al. [19] in Belgium developed a novel AI device (Ghent device) for semen deposition near the uterotubal junction in cattle. The site of semen deposition on fertility was tested with insemination of 12 million sperm deposited in the uterine body using conventional techniques (control) or insemination with a reduced sperm dose (4 million sperm) deposited in the same manner as the control or bilateral deposition near the uterotubal junction using the Ghent device. The authors concluded that neither sperm dosage nor site of semen deposition influenced pregnancy rates.

Intrafollicular insemination is defined as direct introduction of a low-volume (0.06 ml) sperm suspension into a preovulatory follicle. This technique has been recommended as an alternative procedure to the usual deposition of semen into the uterus in cows of low fertility [20]. Even though the results are comparable to intrauterine insemination in a small group of cows, further study is needed to determine its application.

Several methods have been explored to enhance sperm transport following insemination, including administration of prostaglandin (PG)$F_{2\alpha}$ at the time of insemination and clitoral massage immediately after insemination. Administration of a $PGF_{2\alpha}$ analog at the time of insemination improved fertility in heifers compared with non-treated controls, but not in cows [21]. Cooper et al. [22] reported no improvement in pregnancy rates when a five-seconds clitoral massage was done after insemination.

Timing of Insemination

Proper detection of estrus is critical to the success of AI. Standing estrus, or "heat," is the most reliable indication that a cow is in estrus. Studies have shown that a dairy cow displays an average of seven episodes of estrus (stands to be mounted by other cows) [23]. In addition, cows in estrus show estrus activity at any time of the day and hence it is vital that visual observation should be performed several times per day. Increased frequency of visual observation increases the proportion of cows detected in estrus. Several heat detection aids have been developed to assist producers in detection of estrus, including tail paint, Estrus Alert® and Kamar® patches, pedometers, and radio-telemetric systems. A combination of visual observation and one or more of the detection aids increases the effectiveness of estrus detection compared with visual observation or detection aids alone.

Ovulation usually occurs approximately 28–32 hours after the onset of estrus in dairy cows [24]. Optimal fertility of ova is projected to be between 6 and 12 hours after ovulation [25]. The viable lifespan of sperm in the reproductive tract is estimated at 24–30 hours [26, 27]. Given these limitations, the window of opportunity is very short for a successful fertilization.

For several years, researchers have investigated the optimal time to inseminate cows relative to the stage of estrus. Early work led to the establishment of the "a.m.–p.m." recommendation and based on this guideline cows in estrus during morning hours should be inseminated during the afternoon hours, and cows in estrus in the afternoon should be bred the following morning. However, large field trials indicate that maximum conception rates may not be achieved using the a.m.–p.m. rule. The decreased frequency of estrus detection may affect success when AI is performed based on the a.m.–p.m. rule. Highest conception rates for AI occurred between 4 and 12 hours after the onset of estrus in dairy cows [28].

Summary

Accurate insemination technique requires technical and management skills. The level of success ranges from excellent to frustratingly low. Technical skill includes concentration, attention to detail, a clear understanding of reproductive anatomy, and the ability to identify the site of deposition and proper position of the insemination rod. Management skill includes proper semen storage and semen tank management. With scrupulous efforts and attention to detail, the AI program can be successful with farm inseminators. However, successful AI programs depend on not only proper skill and management but also appreciable knowledge regarding risks and pitfalls. Periodic evaluation of AI parameters, such as number of services per conception and conception rate, should be done, with corrective measures taken as required, which may include seeking professional help. Herd inseminators should periodically attend a retraining course to review their techniques and be updated regarding new developments and recommendations regarding AI technique.

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Pharmacological Intervention of Estrous Cycles

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Introduction

Reproductive competence is a major aspect affecting the production and economic success of dairy and beef cattle operations. For herds using artificial insemination (AI), detection of estrus (submission rate) and calving rate are the two major determinants of inter-calving interval. The economic consequences of low efficiency and poor accuracy in the detection of estrus are the main reasons why cattle reproduction research programs focus on developing practical breeding protocols. The important requirements for any effective estrous synchronization protocol are predictable and high estrus and ovulation responses during a specified interval, followed by a higher pregnancy rate to a single insemination carried out in a predetermined time [1]. To achieve better control of the estrous cycle in cattle it is necessary to synchronize emergence of new follicular waves, ensure a luteal phase, terminate the luteal phase, and synchronize ovulation.

Ovarian Follicular Dynamics

Growth of ovarian follicles in cattle occurs in distinct wave-like patterns during the estrous cycle, during pregnancy, and in certain anestrous conditions [2]. Each follicle wave has a lifespan of 7–10 days (range 6–12 days, depending on two vs three waves per cycle and 18 vs 24 days length of cycle) as the follicle progresses through the different stages of development, namely emergence, selection or atresia, and dominance and atresia or dominance and ovulation. Emergence of a new follicular wave is supported by a transient (one to two days) increase in follicle-stimulating hormone (FSH) coinciding with the emergence of a follicular wave. Peak FSH concentrations occur just before wave emergence, and then subsequently decline over several days; concurrently, follicles grow and reach approximately 8.0 mm in diameter [3, 4]. On average, these follicles grow at a constant and comparable rate and then this group of growing follicles is divided into a single dominant and several subordinate follicles. Typically, deviation begins when the diameter of the largest follicles reaches 8.0 mm [3, 4]. The time difference is approximately eight hours in cattle [5]. Once functional dominance is established, the selected dominant follicle continues to grow, while the remaining follicles that emerged during the same follicular wave cease growth and become atretic. Selection of the dominant follicle occurs during declining FSH concentrations; however, the dominant follicle continues to grow (despite FSH at nadir concentrations) until it either ovulates or undergoes atresia [6]. During the final stages of selection of the dominant follicle, there appears to be a transition from mainly FSH to luteinizing hormone (LH) dependency [6]. The selected dominant follicle uniquely expresses mRNA for LH receptors in granulosa cells, which are likely the key to allowing its continued growth under LH stimulation [6, 7]. Estrogen-active dominant follicles are largely LH-dependent; positive feedback of estrogen releases a large amount of gonadotropin-releasing hormone (GnRH), resulting in an LH surge and ovulation of the dominant follicle [6, 7]. Conversely, if the dominant follicle fails to ovulate, the consequences are continued growth of the dominant follicle dependent on increased LH pulse frequency which, if prolonged, can lead to persistence of the dominant follicle [8]. Therefore hormonal treatments that modify both FSH and LH clearly affect the fate of a follicular wave. Manipulation of the follicle wave, in turn, may alter systemic hormonal concentrations, the intrafollicular environment, and the oocyte. Thus, a dominant follicle capable of ovulation is present only at specific times during each wave. Therefore the interval from exogenously induced luteolysis and/or withdrawal of a progestagen treatment to

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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estrus and ovulation depends on the stage of the follicle wave at luteolysis and/or at progestagen withdrawal (whichever occurs last if this does not occur at the same time); cattle with a selected dominant follicle will be in estrus within two to three days, whereas in those in which follicular growth is at the preselection stage, estrus will occur three to seven days later [9–12].

To achieve maximum synchrony of onset of estrus in a synchronization program, it is necessary to have a recently selected dominant follicle present at the end of treatment. Therefore new wave emergence must be synchronized, because both the stage of the follicular wave and the duration of dominance affect the duration of the follicular phase and the interval from treatment to estrus. Synchrony of estrus is optimal when the duration of dominance is either less than 4 days (short) or more than 10–12 days (very long). Further, duration of dominance of the preovulatory follicle can affect fertility. When the duration of dominance of the ovulatory follicle exceeds 10 days, there is a dramatic decline in pregnancy rate [10–12], primarily due to aged oocytes with premature activation. Therefore it is desirable to precisely control follicular dynamics (and in particular new wave emergence) to both minimize the variation in timing of onset of estrus and ensure high pregnancy rates.

Pharmacological Control of New Wave Emergence

The method for controlling follicular wave emergence is based on two principles (Figure 37.1): (i) emergence of a new follicular wave is primarily FSH-dependent; and (ii) an estrogen-active dominant follicle is largely LH-dependent. Thus, the negative feedback effects of progestagens and estrogens can be used to synchronize follicle wave emergence by suppression of FSH and/or LH. However, the extent to which follicles are dependent on gonadotropins during various stages of follicular wave development is poorly defined. Further, details of local mechanisms that play key roles in regulating the sequential progression of follicles through sequential physiological stages of the wave are not well defined. This makes it difficult to develop a simple exogenous hormonal treatment that gives predictable new wave emergence in all animals treated, irrespective of the stage of the follicle wave and stage of estrus at treatment [9, 13].

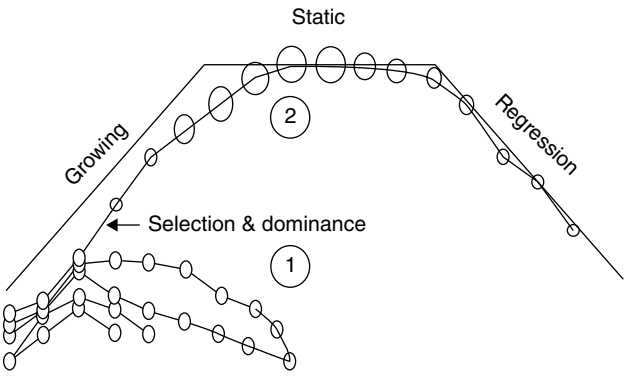


Figure 37.1 Phases of follicular wave. A follicular wave consists of growing, static, and regression phases. The growing phase includes recruitment, and selection and dominance and atresia of subordinate follicles; the static phase includes preparation of ovulation or regression; and the regression phase includes regression of the dominant follicle and preparation for the subsequent wave.

The induction of new follicle wave emergence using exogenous hormonal treatments requires (i) consistent termination of an existing follicle wave, (ii) predictable induction of a transient increase in FSH to induce emergence of a new wave, and (iii) normal growth of the dominant follicle after selection. The primary dependence of the follicle wave progression on gonadotropin support has resulted in the use of steroids to suppress FSH and LH and thus terminate the existing wave. However, it should be realized that FSH and LH are differentially regulated [6]. In the case of FSH, the dominant follicle is the key regulator of the recurrent increases or decreases that occur during the wave. In contrast, although progesterone concentrations are important in regulation of LH pulse frequency, there are changes in LH pulse frequency that occur during the luteal phase which are difficult to clarify by changes in peripheral progesterone or estradiol concentrations. An alternative non-steroidal approach is the administration of GnRH to induce endogenous LH and FSH surges that cause luteinization or ovulation of an existing dominant follicle, thereby inducing emergence of a new wave [14].

Use of GnRH

Functional removal of the dominant follicle or cohort follicles by inducing either ovulation or luteinization using exogenously induced gonadotropin release is a practical approach for induction of a new follicular wave. Administration of GnRH induced an immediate LH and FSH surge, the magnitude of which was independent of progesterone concentration or stage of the follicle wave [15]. However, the effect of GnRH on the existing follicle wave is dependent on the presence or absence of a dominant follicle. GnRH administered after dominant follicle selection caused it to ovulate, with emergence of a new wave 1.5–2.0 days later [14]. However, when GnRH was administered before selection, it had no effect on

progression of the existing follicular wave. In all cows treated after dominant follicle selection, the induced gonadotropin surge was followed by a transient increase in FSH (but not LH) which was associated with new wave emergence [15]. Therefore GnRH synchronizes new wave emergence only when administered in the presence of a functional dominant follicle, whereas if given before dominance it would appear not to affect the subsequent progress of that wave, presumably because of lack of LH receptors on the granulosa cells of such growing follicles. The dichotomy of GnRH effects on progression of the follicle wave is a constraint that needs to be considered when using it as a treatment to synchronize new wave emergence at the start of either progesterone or prostaglandin ($PGF_{2\alpha}$) synchronization regimens [16]. The subsequent use of $PGF_{2\alpha}$ to cause regression of the induced corpus luteum (CL) is mandatory when GnRH is used to synchronize follicle waves. In addition, not only the stage of development of the dominant follicle [17] but also the stage of the estrous cycle [18] at the time that GnRH is administered affects results. If GnRH is administered when the dominant follicle is pre- or post-dominance, ovulation may not occur and a new follicular wave will not emerge [17]. An alternative is to ensure that a viable growing dominant follicle is present at the time of GnRH treatment. In that regard, cattle will respond most consistently when GnRH is administered between days 5 and 12 of the estrous cycle [18, 19].

Use of Estradiol

Even though combined treatment with progestin and estradiol has been utilized for decades, knowledge of the physiological effects and mechanisms of action of exogenous estradiol and progesterone on follicles is a prerequisite to the development of improved methods of controlling the estrous cycle. Estradiol suppresses the growing phase of the dominant follicle; suppression is more profound when given in combination with progesterone [20]. The mechanism responsible for estrogen-induced suppression of follicle growth appears to involve suppression of both FSH and LH [21]. Further, it exerts its negative effect on both the hypothalamus and pituitary. Studies have focused on the effects of short-term estradiol and progesterone treatments, either alone or in combination at the time of pre-follicle wave emergence, on LH and FSH, progression of the follicle wave, and changes during follicle wave development [1]. The period of elevated FSH associated with wave emergence was shortened by approximately 10 hours following estradiol, progesterone, or a combination of estradiol and progesterone treatments, compared with control. Frequency of LH pulses was reduced following progesterone treatment and was further reduced in response to a combination of estradiol and progesterone treatments. This steroid combination also significantly reduced pulse amplitude. The emergence of the follicle wave and selection of the first dominant follicle of the cycle were delayed approximately 1.5 and 2.5 days, respectively, by the combination of estradiol and progesterone treatment. Intrafollicular estradiol concentrations were suppressed in the largest follicle following combination estradiol and progesterone treatment, whereas under estradiol treatment they were unchanged. However, they were suppressed in smaller follicles following both estradiol and combination estradiol and progesterone treatments. Therefore suppression of LH pulses by the combination estradiol and progesterone treatment is important for suppressing follicular estradiol in the largest follicle, whereas suppression of FSH alone may be sufficient to reduce follicular estradiol concentrations in smaller follicles.

In estrous synchronization protocols, estradiol is usually administered at the initiation of the protocol, concurrent with insertion of a progestin-releasing device. The combination of estradiol and progestin was used to determine if suppression of follicle growth would induce new wave emergence at a consistent interval after treatment regardless of the phases (i.e. growing, static, or regressing phases) of follicle development at which treatment was initiated. The use of estradiol-17β in progestin-implanted cattle was followed consistently by the emergence of a new wave, on average 4.5 days later [9, 16]. Once the estradiol was metabolized, there was an FSH surge and a new follicular wave emerged [22, 23]. The administration of 2.5 or 5 mg estradiol-17β (or 2 mg estradiol benzoate or estradiol valerate) in progestin-implanted cattle at random stages of the cycle was followed by the emergence of a new follicular wave approximately four days later, with little variability [24–26].

The mechanism of action of estradiol would appear to be through FSH and LH, causing follicle atresia followed by synchronous release of FSH and emergence of a new follicle wave. Hence, in general, estradiol has been the hormone treatment used most successfully to synchronize follicle wave emergence in cattle. However, the use of estradiol is prohibited in an increasing number of countries, so alternatives must be sought. Similarly, the use of GnRH or LH to induce ovulation, and to remove the suppressive effects of a dominant follicle on FSH, result in a surge in FSH and a new follicle wave at a predictable time thereafter. Therefore synchronization of follicle wave emergence is likely to be through FSH release, which may involve manipulation of ovarian (follicular) metabolic pathways or feedback mechanisms to the hypothalamic–pituitary axis.

Controlling the Lifespan of the Corpus Luteum

Shortening the Luteal Phase (Premature Luteolysis)

Luteolysis is initiated by estradiol from the developing preovulatory follicle, which triggers the release of hypophyseal oxytocin, which in turn stimulates release of a small quantity of uterine $PGF_{2\alpha}$. $PGF_{2\alpha}$ then initiates a positive feedback loop involving release of additional luteal oxytocin and $PGF_{2\alpha}$ of both luteal and uterine origin [27]. Oxytocin stimulates synthesis and secretion of $PGF_{2\alpha}$ from the uterus. It has recently been proposed that release of luteal $PGF_{2\alpha}$ amplifies the luteolytic signal in an autocrine or paracrine manner [27, 28]. $PGF_{2\alpha}$ causes decreased blood flow to luteal parenchyma and morphological changes that reduce both the number of small steroidogenic luteal cells and the size of large luteal cells. In addition, it is involved in intracellular signaling that facilitates apoptosis of large luteal cells and stimulates intraluteal production of $PGF_{2\alpha}$, thereby resulting in the demise of the CL [29, 30].

Since the discovery of $PGF_{2\alpha}$ as the luteolytic agent in cattle [31, 32], it has been the most commonly used treatment for elective induction of luteal regression and/or synchronization of estrus [33]. The injection of $PGF_{2\alpha}$ causes immediate regression of the CL after approximately day 5 of the estrous cycle: progesterone concentrations decline rapidly to basal levels within 24 hours, and LH pulse frequency increases, causing a significant increase in estradiol from the dominant follicle and the induction of estrus and ovulation. Despite rapid luteolysis, the interval from treatment to the onset of estrus is variable and dependent on the stage of the follicle wave at treatment. Animals with a functional dominant follicle are typically in estrus within two to three days, because the dominant follicle at the time of induced luteolysis ovulates shortly (Figure 37.2a); however, animals at the pre-dominance or regression phase of the wave will require four to six days to form a dominant follicle and hence have a longer and more variable interval to onset of estrus (Figure 37.2b).

$PGF_{2\alpha}$ fails to effectively induce luteolysis during the first five or six days following estrus. Lack of responsiveness was due to lack of $PGF_{2\alpha}$ receptors in immature CL, but recent work has demonstrated the presence of $PGF_{2\alpha}$ receptors in the CL as early as two days after ovulation [34]. However, it appears that the mature CL may possess a positive feedback loop that results in intraluteal $PGF_{2\alpha}$ production that may continue the process of luteolysis initiated by a single treatment of exogenous PGF [35]. It should be noted that although luteolytic response was variable when a single dose of $PGF_{2\alpha}$ was given on days 5 or 6 of the estrous cycle, two doses of prostaglandin analog (12 hours apart) consistently induced luteal regression (95–100%) at two to five days after estrus [36, 37].

Extending/Simulating a Luteal Phase with Exogenous Progesterone

Development of intravaginal progesterone delivery devices, such as the progesterone-releasing intravaginal device (PRID, 1.55 g), controlled intravaginal releasing device [CIDR, 1.38 g (US) or 1.9 g (Canada)], and Cue-Mate (1.56 g) intravaginal progesterone device (IPVD), has facilitated the use of progestagens for synchronization in cattle. In general, for all progesterone-based synchronization treatments, a high proportion (up to 85%) of cows exhibit estrus 36–72 hours after removal. This higher degree of estrous synchrony allows for fixed-time AI (FTAI) between 48 and 84 hours after removal, with hormonal induction of ovulation using GnRH or estradiol. Alternatively, cows may be observed for estrus and inseminated once after detection of estrus.

Estrous synchronization systems that include progesterone in the protocol have three primary advantages. First, maintaining the concentration of progesterone at greater than threshold level (>1 ng/ml) suppresses the LH surge and estrous behavior. Treatment with progesterone for 14 or 21 days resulted in a high estrus response within three days after progesterone removal [38]. However, conception rates following prolonged progesterone treatment are 10–15% below those of cows treated with progesterone for shorter duration. The reason for low conception is that intravaginal progesterone releasing devices result in progesterone concentrations that are lower than those of the luteal phase, and LH pulse frequency and peripheral estradiol concentrations are elevated. Consequently, the dominant follicle continues to receive gonadotropin support beyond the time where either ovulation or atresia would have occurred. Ultrastructural changes in the oocyte within the persistent dominant follicle are believed to reduce fertility of treated cows [10, 39]. Fertility concerns for progesterone-treated cows have led to development of short-term protocols (seven to nine days) for progesterone use. Progesterone is also typically combined with follicular synchronization so that a newly developing dominant follicle is present during progesterone supplementation. Progesterone supplementation is an effective method for treating anestrus, which affects approximately 20% of beef cows at the start of the breeding season [40, 41]. Furthermore, in US dairies, 28% of primiparous cows and 15% of multiparous cows had not ovulated by 60 days after calving [41]. Thus the second primary advantage for progesterone in estrous synchronization protocols is that

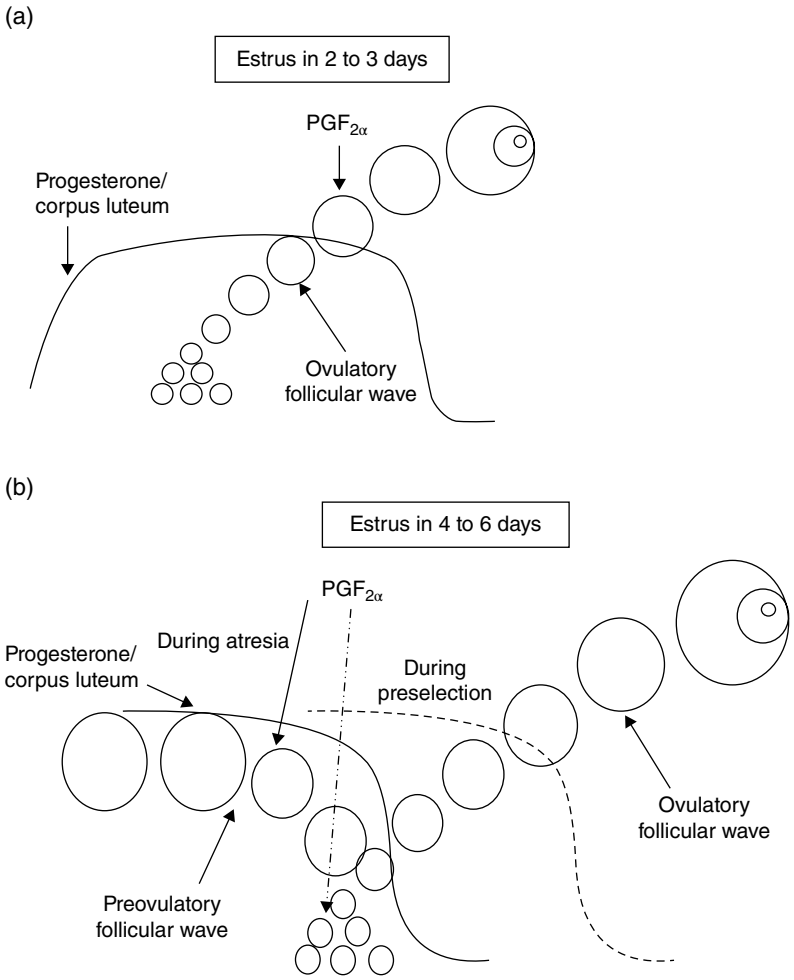


Figure 37.2 Interval from $PGF_{2\alpha}$ administration to estrus. (a) Animals with a functional dominant follicle are in estrus within two to three days because the dominant follicle at the time of induced luteolysis ovulates soon thereafter. (b) Animals at the pre-dominance or regression phase of the follicular wave will require four to six days for a dominant follicle to reach preovulatory diameters and hence have a longer and more variable interval to onset of estrus.

anestrous cows are responsive when progesterone is used. Progesterone supplementation in an anestrous cow decreases LH initially, but then increases LH pulsatility [42, 43]. The increase in LH pulsatility increases dominant follicle development. Progesterone also primes the hypothalamic center for estrous expression and the LH surge. Removal of the progesterone is followed by estrus, ovulation, and a normal-length luteal phase in a large percentage of treated cows [42]. Treatment of cystic ovarian disease is the third advantage of progesterone supplementation. Approximately 10% of US dairy cows are affected [44]. Abnormally high LH pulsatility and absence of an LH surge are characteristic endocrine features of the disease [44–46]. Progesterone supplementation decreases LH pulsatility in cystic cows. The decrease in LH pulsatility after progesterone treatment is followed by turnover of the cystic (dominant) follicle. Development of a new dominant follicle is normal and ovulation occurs after progesterone withdrawal [45–47].

Induction of Ovulation

Prior to the preovulatory LH surge in cyclic cows, the frequency of LH pulses gradually increases to one pulse per hour in response to endogenous GnRH pulses. In cyclic cows a single dose of exogenous GnRH induces LH release. Therefore a single injection of GnRH induces an LH surge and ovulation when given at approximately days 10–18 after calving in dairy cows and days 21–31 after calving in beef cows [41]. A single injection of GnRH combined with 48-hour calf removal also induces ovulation in beef cows. A single injection of human chorionic gonadotropin (hCG) 500–3000 IU induces ovulation in dairy and beef cows. Further, because of its positive feedback effect on the

surges of FSH and LH, exogenous estradiol has been used to induce ovulation in cows. It should be noted that the GnRH-induced CL in postpartum cows usually has a shorter lifespan than a spontaneously formed CL. Further, administration of GnRH prior to complete uterine involution results in pyometra in postpartum dairy cows. Therefore a single injection of GnRH during the early postpartum period is not very useful in practice.

Synchronization of Estrus

Prostaglandin Protocol

The mechanism of action of prostaglandin is luteolysis, and thus the application of a prostaglandin program is restricted only to cycling cows with a functional mature CL. The administration of a luteolytic dose of $PGF_{2\alpha}$ (25 mg) or its synthetic analogs (e.g. 500 µg cloprostenol) causes regression of the CL in most stages of diestrus, thereby inducing estrus in over half (55–65%) of a cycling herd (Figure 37.3a). A second injection given 11–14 days later should result in synchronized estrus of the herd because the cows that responded to the first injection will be on day 6–15 of diestrus, with the majority being on days 7–9 (Figure 37.3b). A 100% response is not realistic, even if every cow in the group was known to have a mature CL at the time of the second injection. This is partly because not all mature corpora lutea, at least in lactating dairy cattle, are lysed by $PGF_{2\alpha}$. For example, approximately 25% of the cows that were on day 6 respond, approximately 33% of those that were on day 7 respond, and fewer than 100% of those that were on days 8–16 of the cycle respond. Many prostaglandin-controlled synchronization programs have been developed (Figure 37.3). The selection of a program best suited to a herd depends on a variety of factors including efficiency of estrus detection on the farm, palpation skills of the attending veterinarian (Figure 37.3c), cost of treatment, availability of labor, and the objectives of the program. Further, these programs involve intramuscular injections of a single luteolytic dose of $PGF_{2\alpha}$. Various doses and injection sites have since been compared involving the ischiorectal fossa (one luteolytic dose) and vaginal submucosa (half a standard luteolytic dose). Administration of half a standard luteolytic dose submucosally in the vagina or injection of a single luteolytic dose of $PGF_{2\alpha}$ into the ischiorectal fossa was as efficacious as intramuscular administration [48].

Progesterone Protocols

Fourteen-Day Treatment

In this protocol, cows are supplemented for 14 days usually using an IVPD or oral progestin (melengestrol acetate, MGA) (Figure 37.4). Since ovulation is inhibited for up to 14 days, the follicle that ovulates following this protocol is aged (from a persistent follicle) and hence has reduced fertility. Therefore $PGF_{2\alpha}$ is given 19 days following progesterone/progestin removal. The animals are observed for two to four days following treatment with $PGF_{2\alpha}$ and inseminated at observed estrus using the a.m.–p.m. rule. This injection of $PGF_{2\alpha}$ regresses the CL that forms after the

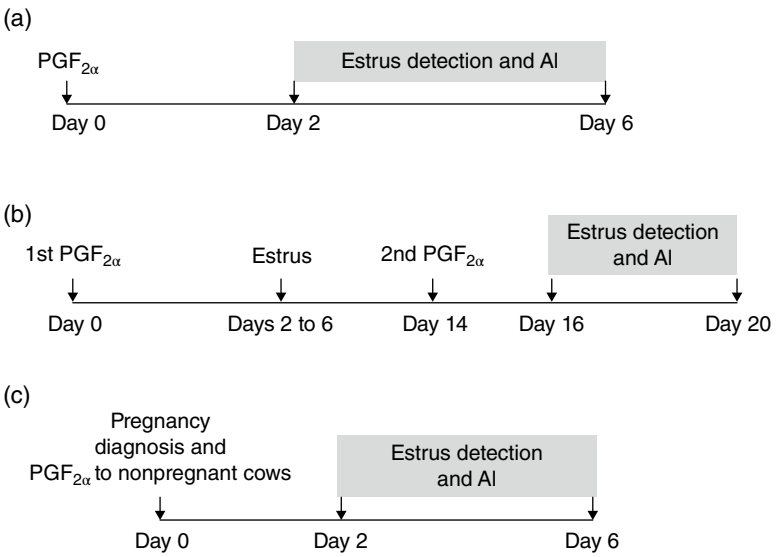


Figure 37.3 Synchronization of estrus using $PGF_{2\alpha}$. (a) Single $PGF_{2\alpha}$ program. (b) Double $PGF_{2\alpha}$ program. (c) Administration of $PGF_{2\alpha}$ to non-pregnant cows at pregnancy diagnosis. Note that under field conditions, the pregnancy examination is performed by a veterinarian to avoid iatrogenic pregnancy loss.

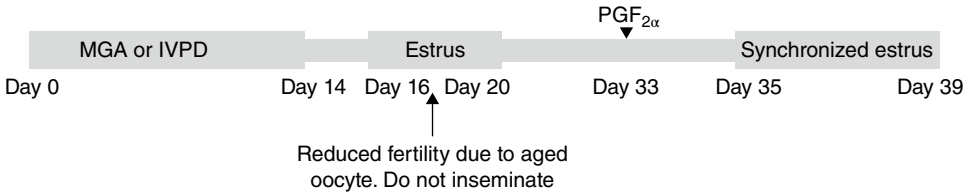


Figure 37.4 Estrus synchronization protocol with progesterone supplementation for 14 days using an IVPD or MGA and $PGF_{2\alpha}$.

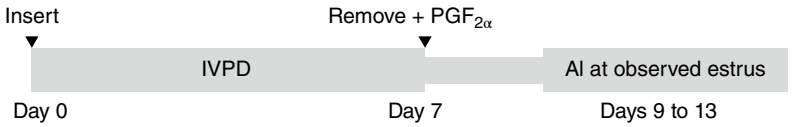


Figure 37.5 Estrus synchronization protocol with progesterone supplementation for seven days using an IVPD and $PGF_{2\alpha}$.

ovulation of a persistent follicle, and an estrus with good fertility will occur. This type of synchronization protocol with longer duration of progesterone treatment (14–17 days [49] and 14–19 days [50, 51]) has become a commonly used method of synchronizing estrus in replacement beef heifers. MGA, an orally active progestin [52], is capable of inhibiting estrus and ovulation in heifers when consumed at 0.5 mg/head daily for 14 days in a supplement carrier. Usually MGA is mixed with grain or a protein supplement and fed to cattle. The MGA program is usually a cost-effective synchronization program. It is important to ensure that all animals enrolled receive the recommended dose. Recently, a 14- to 16-day IVPD–$PGF_{2\alpha}$ protocol was evaluated for synchronization of estrus in replacement beef heifers in an attempt to replace the oral MGA program [53, 54]. This has the potential to improve synchrony of estrus compared with other protocols currently available. Progesterone has been shown to effectively presynchronize estrus [49, 54, 55] in replacement beef heifers and induce ovulation in heifers that did not reach puberty before administration of treatments [56].

Seven-Day Exposure

In this synchronization program, progestin, usually an IVPD, is used for seven days (Figure 37.5). Progesterone from the device mimics the progesterone produced by the CL and inhibits estrus and ovulation. When ovulation is inhibited for seven days, all cycling animals are expected to have a CL that is at least seven days old at $PGF_{2\alpha}$ treatment. Therefore all animals with a CL will respond to $PGF_{2\alpha}$. Animals in which a CL had regressed during the seven-day period will show standing estrus following removal of the progestin. Estrus is observed two to six days after removal of progesterone; those detected in estrus are inseminated using the a.m.–p.m. rule (cows first detected in estrus in the morning will be inseminated the same evening and cows first detected in estrus in the evening will be inseminated the next morning).

Use of GnRH in a Seven-Day Protocol

Progesterone exposure will not synchronize follicular waves. However, exogenous GnRH at the start of progesterone treatment will synchronize follicular waves. The normal duration of a follicular wave is 7–10 days; therefore if a mature follicle is present at the initiation of the protocol, that follicle will persist (and ovulate an oocyte with reduced fertility). Initiation of a new follicular wave at the beginning of the protocol by administering GnRH will reduce the occurrence of any persistent follicle forming. Furthermore, the progestin that is present between the GnRH and $PGF_{2\alpha}$ injections will virtually eliminate the chance of animals exhibiting signs of standing estrus before the $PGF_{2\alpha}$ injection (Figure 37.6). The animal is inseminated at observed estrus following progesterone removal.

Synchronization of Ovulation

Synchronization of ovulation involves a combination of GnRH and $PGF_{2\alpha}$, with or without progesterone supplementation during the course of the treatment. These protocols facilitate insemination at a predetermined (fixed) time without the need for detection of estrus. Several protocols have been successfully employed with acceptable pregnancy rates.

GnRH–$PGF_{2\alpha}$–GnRH Protocol

The protocol involves an injection of GnRH at a random stage of the estrous cycle, followed by an injection of $PGF_{2\alpha}$ seven days later. A second injection of GnRH follows the $PGF_{2\alpha}$ injection by 48 hours. The method is designed to be used with timed insemination 16 (range 8–24) hours after the last GnRH injection (Figure 37.7a; Ovsynch) [14]. This synchronization system results in a tight synchrony of

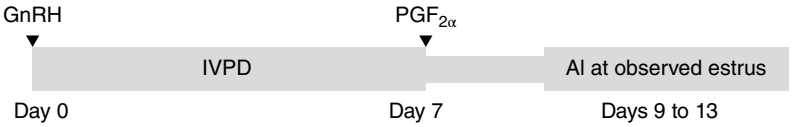


Figure 37.6 Estrus synchronization protocol with progesterone supplementation for seven days using an IVPD, GnRH, and $PGF_{2\alpha}$. Note that the addition of GnRH is to initiate a new follicular wave. GnRH is replaced with estradiol-17β or estradiol benzoate plus progesterone.

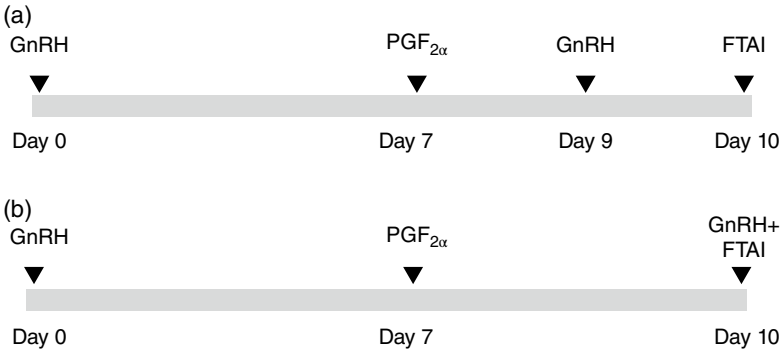


Figure 37.7 Ovulation synchronization protocol using GnRH–$PGF_{2\alpha}$–GnRH for a predetermined timed insemination (FTAI). (a) Ovsynch protocol. (b) CO-Synch protocol. Note that the first GnRH is replaced with estradiol-17β or estradiol benzoate plus progesterone and the second GnRH with estradiol-17β or estradiol benzoate. The concurrent administration of GnRH at the time of insemination in the CO-Synch protocol is preferred in beef farms as it reduces number of handlings.

estrus, allowing breeding at an appointed time without detection of estrus. The first injection of GnRH causes either ovulation or luteinization of all dominant or large growing follicles. As a result, a new follicular wave is initiated in all cows by approximately three days after the injection. Therefore all the females in the group have growing follicles of about the same stage of development. In addition, GnRH stimulates development of luteal tissue from the cells that were previously the dominant follicle. The $PGF_{2\alpha}$ injection lyses the CL resulting from the initial GnRH injection. Furthermore, the final GnRH injection serves to increase the synchrony of ovulation within a group of cows. The success of the first injection of GnRH to synchronize follicular growth is very good in cows, but less so in heifers. The CO-Synch protocol is similar to the Ovsynch protocol, except that the second injection of GnRH is given concurrent with AI (Figure 37.7b). This has a huge advantage in beef synchronization regimens because the CO-Synch regimen requires one less handling.

Presynch Protocol

It is evident that stage of estrous cycle at initiation of the Ovsynch protocol influences the success of synchrony of the follicular wave. Initiation of the protocol too early (e.g. one to four days after ovulation) or too late (e.g. 13–20 days after ovulation) reduced synchrony. Furthermore, if the first GnRH treatment was given when the dominant follicle was pre- or post-dominance, ovulation may not occur and synchronous emergence of a new follicular wave will not occur [17]. An alternative is to ensure that a viable growing dominant follicle is present at the time of GnRH treatment. Cattle will respond most consistently when GnRH is given between days 5 and 12 of the estrous cycle [18, 19]. Consequently, several protocols have been employed to align cows at days 5–12 of the estrous cycle at initiation of an Ovsynch or CO-Synch protocol, including presynchronizing cows before Ovsynch using two injections of $PGF_{2\alpha}$ administered 14 days apart, with initiation of the timed AI protocol 11 days [57], 12 days [19], or 14 days [58] later (Presynch-Ovsynch). Another protocol, designated G6G, starts with an injection of $PGF_{2\alpha}$, which is intended to induce luteolysis of functional corpora lutea, and a GnRH injection two days later, intended to induce an ovulation. Collectively, the two injections of G6G are intended to initiate a new estrous cycle. The Ovsynch is scheduled to start six days after the GnRH injection in order for the cow to be on day 6 of a new estrous cycle and very likely to have a functional dominant follicle, capable of ovulating in response to the first GnRH of Ovsynch. Further, use of two injections of $PGF_{2\alpha}$ to presynchronize estrous cycles was not effective for anovular cows [19]. Presynchronization protocols that induce production of a CL before initiation of the Ovsynch protocol are likely to improve fertility in anovular cows. A protocol that involves induction of cyclicity in anovular dairy cows is the use of an Ovsynch protocol to presynchronize and induce cyclicity in cows before the use of a breeding Ovsynch protocol, termed Double-Ovsynch [59].

Addition of Progesterone in GnRH–$PGF_{2\alpha}$–GnRH Protocol

Progesterone supplementation between the first GnRH and $PGF_{2\alpha}$ for seven days in Ovsynch (Figure 37.8a) and CO-Synch (Figure 37.8b) protocols enhances estrus/ovulation synchrony and conception in cows and heifers. These protocols are advantageous in anestrous cows and heifers and in cows with cystic ovaries. Several protocols are available for use, modified from the above-mentioned protocol. Recently, progesterone supplementation between the first GnRH and $PGF_{2\alpha}$ for five days in CO-Synch protocol (Figure 37.8c) has been evaluated. The objective is to shorten progesterone treatment to five days and to increase the proestrus phase (the interval from $PGF_{2\alpha}$ to the second GnRH) to 72 hours [60, 61]. This approach increased AI pregnancy rates up to 15% in beef cows [40, 41]. Because of the shortened interval from the initial GnRH to $PGF_{2\alpha}$ in the five-day IVPD protocol, and variability in luteolysis in some cows when a single dose of $PGF_{2\alpha}$ is given on day 5 of the estrous cycle, all cows should receive a second dose of $PGF_{2\alpha}$ treatment approximately 6–12 hours after the first $PGF_{2\alpha}$ given at CIDR withdrawal [62, 63].

Addition of Equine Chorionic Gonadotrophin

Equine chorionic gonadotropin (eCG) is added to the synchronization treatment protocols as a means of increasing follicular development. The utilization of eCG in breeding protocols improves reproductive efficiency in seasonally calving, anestrous cattle. Addition of 400 IU eCG at progesterone device removal resulted in 74% pregnancy in a 49-day breeding season [64]. Inclusion of eCG in 6 or 7 days of the GPG/P4 protocol increased pregnancy in a 28-day breeding (58.6 vs 52.3%) and decreased median days to conception in anestrous dairy cows [65]. In South American pasture-based lactating beef and dairy lactating anestrous cows, addition of eCG increased pregnancy rates by 10–15% after FTAI compared with the same protocol without eCG [66–68].

Split-Time AI

A proportion of females undergoing estrus synchronization do not express estrus prior to FTAI. To account for these non-estrous females, all cows and heifers are administered GnRH at FTAI to ensure that ovulation is induced. However, pregnancy rates are on average 27% lower among females that fail to express estrus prior to FTAI [69]. Researchers at the University of Missouri developed an alternative approach to manage timed insemination of cows and heifers that have not expressed estrus prior to FTAI. This approach, known as "split-time AI" (STAI), delays insemination of non-estrous females until 20–24 hours after the scheduled timed AI. AI

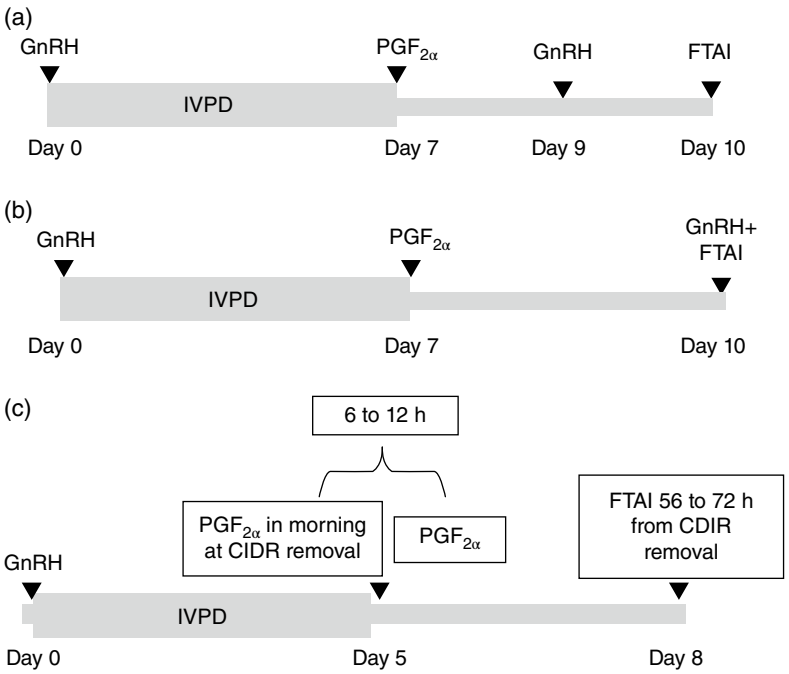


Figure 37.8 Ovulation synchronization protocol using GnRH–$PGF_{2\alpha}$–GnRH with addition of progesterone for a predetermined timed insemination (FTAI). (a) Ovsynch with IVPD. (b) CO-Synch with IVPD. (c) Five-day CO-Synch with IVPD. Note that the first GnRH is replaced with estradiol-17β or estradiol benzoate plus progesterone and the second GnRH with estradiol-17β or estradiol benzoate. The addition of progesterone is considered advantageous in anestrous cows and prepubertal and peripubertal heifers. Concurrent administration of GnRH at time of insemination in CO-Synch + IVPD protocol is preferred on beef farms as it reduces the number of handlings.

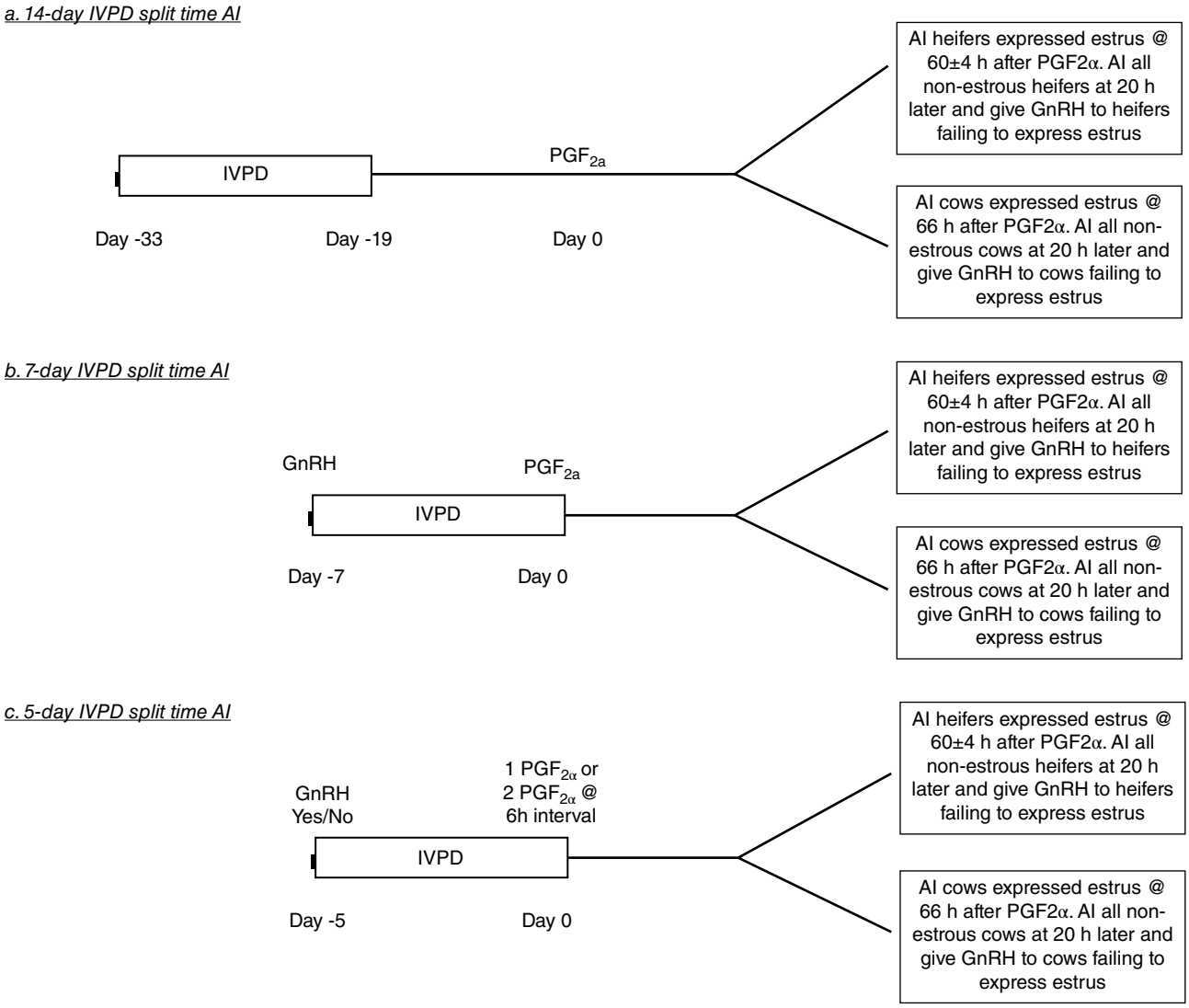


Figure 37.9 STAI protocol using GnRH–$PGF_{2\alpha}$–GnRH with addition of progesterone for a predetermined timed insemination (FTAI) for cattle expressing estrus and delayed AI for cattle that failed to express estrus. (a) Fourteen-day STAI with IVPD. (b) Seven-day STAI with IVPD. (c) Five-day STAI with IVPD. Note that due to the shortened interval from initial GnRH to $PGF_{2\alpha}$ in the five-day IVPD protocol and variability in luteolysis, when GnRH is administered at the time of IVPD all beef females should receive a second dose of $PGF_{2\alpha}$ approximately 6–12 hours after the first $PGF_{2\alpha}$ given at IVPD withdrawal on day 5, and when GnRH is not administered at the time of IVPD all dairy females should receive one dose of $PGF_{2\alpha}$.

pregnancy rates can be optimized through use of a STAI approach following administration of the 14-day IVPD-$PGF_{2\alpha}$ or 5-day CO-Synch protocol for heifers and the 7- or 5-day CO-Synch + IVPD protocol for mature cows (Figure 37.9). Estrus detection aids applied at the time of $PGF_{2\alpha}$ administration allow producers to determine the estrous status of females and inseminate at the optimal time. STAI improved timed AI pregnancy rates among beef replacement heifers following the 14-day IVPD-$PGF_{2\alpha}$ protocol [70] and among mature beef cows following the 7-day CO-Synch + IVPD protocol [71, 72]. Typically, 30–50% of mature cows and 20–40% of heifers fail to express estrus prior to 66 hours after prostaglandin following the 7-day CO-Synch + IVPD and 14-day IVPD-$PGF_{2\alpha}$ protocols, respectively. Results from the University of Missouri study showed that 55% of both heifers and cows that had failed to express estrus by 66 hours after $PGF_{2\alpha}$ went on to express estrus by the time delayed AI was performed. The pregnancy rates of non-estrous females were improved 16 percentage points by delaying insemination by 20–24 hours [70, 72]. Further, in addition to offering improved pregnancy rates compared to FTAI, STAI allows for a reduction in the use of GnRH. Since administration of GnRH was not found to be required for cows or heifers with activated estrus detection

patches at AI [73], insemination can be performed without GnRH administration for cows and heifers that have expressed estrus prior to the standard time of appointment breeding, as well as for cows and heifers that expressed estrus prior to the delayed time point.

Summary

Estrous and ovulation synchronization protocols now exist to successfully achieve acceptable fertility after applying insemination at observed estrus or at a predetermined time without estrus detection. Further, success is achieved in females that may not be cycling at the onset of treatment. Initiation of fertile ovulation in peripubertal heifers and acyclic cows is likely the primary manner in which producers may improve fertility in response to estrous synchronization and timed AI protocols. Use of IVPD-based synchronization before insemination of cows and heifers enhances fertility more effectively than other hormonal synchronization interventions. Development of synchronization protocols that reduce the factors associated with reducing synchrony of estrus and/or ovulation will provide cattle producers with efficient and effective tools for improving genetics in their operations. Since location, pasture difference, diet, breed composition, body condition, postpartum interval, climate, and geographic location may affect the success of synchronization protocols, these variables should be kept in mind to enhance the success of synchronization.

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38

Examination for Pregnancy: Rectal Palpation

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Introduction

Pregnancy diagnosis is an invaluable tool to the cattle producer who is trying to maintain maximum profitability. In the case of the beef cattle herd, a significant portion of the cost of producing calves is due to the feeding and care of the pregnant dam particularly during the winter months. Cows that are open (not pregnant) will add to the cost of maintaining the herd while not providing income in support of the producer. Similarly, in dairy cattle, a cow that fails to become pregnant in a timely fashion will ultimately produce less milk for the dairyman, thus adding cost but not enhancing income. A pregnancy test that would identify pregnant and open cows before they return to estrus, thus allowing intervention and rebreeding by the subsequent estrus, would help minimize the calving interval and thereby maximize profit especially in a program utilizing artificial insemination. In addition, the ideal pregnancy test would be minimally invasive, simple to perform requiring little investment in equipment, supplies, and time, yield quick results allowing cow-side decisions to be made by the producer, and have high sensitivity and specificity. Finally, in many herds the capability to estimate the stage of gestation, the gender of the fetus, and the presence of pathologic conditions of the reproductive organs can be very helpful.

Currently there are three reliable methods of diagnosing the pregnancy status of a cow: transrectal palpation, transrectal ultrasound examination, and endocrine testing (Table 38.1). While none of these meets all the criteria for the ideal test, each has its advantages and disadvantages. The producer and veterinarian must carefully consider each test and determine which provides the most benefit for the conditions at hand. Of these methods, transrectal palpation is the most commonly utilized and meets the most criteria under the most circumstances.

The diagnosis of pregnancy by transrectal palpation is a skill that can readily be acquired and honed to great sensitivity and specificity with good understanding of normal anatomy and physiology of the cow. A good understanding of pregnancy and fetal development also allows the examiner to accurately estimate the stage of pregnancy with experience. A thorough discussion of normal anatomy, physiology, and pregnancy can be found in previous chapters. With this understanding and practice, one can achieve very consistent results approaching 100% accuracy as early as day 30–35 of pregnancy. Transrectal examination of the reproductive tract begins with identification of the cervix which is fibrocartilagenous and firm (sometimes described as feeling somewhat like a chicken neck) and serves as the landmark for the reproductive organs. In the non-gravid or early pregnant uterus, it typically is found within or just at the brim of the pelvis. In multiparous cows and as pregnancy progresses, it may be found pulled more cranial and ventral. It is important to remember that the cervix is relatively mobile and one may have to sweep the pelvic canal and work over the brim of the pelvis until it is found. Once identified, the cervix may be grasped and used to retract the body, horns, and uterus caudally to allow better access to and examination of these structures. Then you can follow the cervix cranially to the body of the uterus and identify the bifurcation of the horns. At this location, the ventral intercornual ligament (see Figure 22.6) can be used to retract the horns and lay them along the floor of the pelvis to facilitate thorough palpation of each horn. Just beyond the tip of each horn, the ovaries can be palpated. As pregnancy progresses, the size and weight of the fetus and fetal fluids will make retraction of the cervix and uterus impossible and they will have to be examined in place.

The definitive diagnosis of pregnancy is based on the identification of structures that can only occur if the cow is

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Table 38.1 Comparison of different methods of pregnancy diagnosis.

Criteria	Endocrine testing	Ultrasound examination	Transrectal palpation
Allows intervention prior to first estrus after breeding	Yes	No	No
Minimally invasive	Minimal to moderate	Yes	Yes
Simple to perform, little investment/cost	Moderate to high	Moderate	Simple, minimal cost
Results available quickly	Minutes to days	Instant	Instant
Estimates stage of gestation	No	Yes	Yes
Gender determination	No	Yes	No
Identifies pathologic conditions of reproductive tract	No	Yes	Yes
Sensitivity/specificity	Varies (see Chapter 39)	Excellent	Excellent

indeed pregnant; if none of these can be identified, the cow should be considered not pregnant or the diagnosis deferred. Four such structures can be readily palpated and are considered the cardinal signs of pregnancy. They are chorioallantoic (membrane) slip, the amniotic vesicle, placentomes, and the fetus. A diagnosis of not pregnant should not be made until complete palpation of the body and both uterine horns is performed.

In addition, other changes in the reproductive tract can be palpated that are considered supportive of a positive diagnosis of pregnancy. These include the inability to retract the cervix, fluid within or fluctuance of the uterus, enlarged diameter and asymmetry of the uterine horn(s), and enlargement and fremitus (a buzzing sensation) of the middle uterine artery.



Figure 38.1 Slipping of the chorioallantoic membrane. *Source:* Courtesy Maarten Drost, VISGAR.

Cardinal Signs of Pregnancy

Membrane Slip

Membrane slip can be palpated routinely by 30–32 days of pregnancy in the gravid horn (as early as 28 days in heifers as one gains experience) and in both horns by day 50–60 of gestation. Following identification of the cervix and retraction of the uterus, each horn can be grasped gently between the fingers and thumb and lifted slightly (Figure 38.1). If pregnant, a distinct popping sensation can be felt as the membrane slips from the grasp within the uterine walls. At 32 days of pregnancy it gives the impression of a thread and by day 45 a small string slipping from the fingers in the gravid horn. By day 60 it is palpable in both horns as a somewhat larger string and by day 75 is approaching the feel of a piece of yarn slipping from the fingers. Membrane slip is most commonly used up to day 90 of gestation.

Amniotic Vesicle

The amniotic vesicle is a very turgid, fluid-filled sack surrounding and protecting the embryo proper during early gestation. It can be palpated as a small knot or bump within the uterine horn approximately 6–7 mm in diameter by 32–35 days (5 weeks) of gestation. By 6 weeks of gestation it will be 1.5 cm in diameter and at 7 weeks it will be 3.5–5 cm in diameter. By 8 weeks, the vesicle will be 6–7 cm and will be losing its turgidity, generally allowing for palpation of the fetus proper around day 60. As this occurs, the vesicle itself will become more difficult to identify and can generally no longer be used to determine pregnancy.

Fetus

As the fetus becomes palpable around day 55–60, it will be approximately 5–6 cm in length and can be felt floating in the fluid of the gravid horn by carefully cupping the horn with the fingers and sliding them along the length of the

pregnant horn. Alternatively, the wall of the uterus may be lightly tapped by the fingers, creating a wave of fluid and causing the fetus to bump back against the fingers (referred to as ballottement). As the pregnancy advances, fetal parts may more readily be identified such as the head, feet, and legs, allowing the beginner to be more comfortable with the diagnosis. By three months of pregnancy, the uterus and fetus will reach sufficient size and weight to cause it to drop over the brim of the pelvis and into the abdominal cavity and by four to five months of pregnancy the fetus may descend into the abdomen far enough to make palpation of the fetus itself difficult or impossible, particularly in large, deep-bodied cows. Usually by the seventh month the fetus has grown large enough that it will be readily accessible and can once again be used to determine pregnancy status.

Placentomes

Placentomes (occasionally referred to as buttons by lay personnel) are made up of the maternal caruncle and fetal cotyledon. With experience they may be palpated as early as 75–80 days of gestation as soft pea-sized swellings or bumps on the internal uterine wall, but are a bit more firm and readily palpated by day 90. At this time they are about the size of a dime and will reach about the size of a half dollar by the time the cow is five months pregnant. During mid-gestation as the fetus becomes more difficult to reach, placentomes may become the best method to definitively diagnose pregnancy. At least three should be identified, ensuring that the ovaries are not mistaken for placentomes.

Supportive Signs of Pregnancy

As pregnancy advances to 60 days and beyond, the weight of the fetus and fetal fluids pulls the cervix cranially over the edge of the pelvis and into the abdomen. It becomes increasingly difficult at this time to retract the uterus into the pelvic canal; thus, if the cervix feels "heavy" and relatively immobile, pregnancy can immediately be suspected and the palpator can search for the cardinal signs found in more advanced pregnancy such as the fetus and placentomes. This can save time and effort that would be required to find the more subtle membrane slip or amniotic vesicle.

Fluid within or fluctuance of the uterus is sometimes used in early pregnancy (as early as 28 days) to make a presumptive diagnosis of pregnancy, but the owner must clearly understand that it is presumptive only and should be confirmed at a later date. The wall of the pregnant uterus at this time thins significantly, resulting in a structure that feels much like a partially filled water balloon. This fluid, along with the developing fetus, results in an enlarged diameter of the gravid uterine horn, and later, as the pregnancy progresses, both uterine horns. This asymmetry early in gestation and the enlarged uterine horn(s) are highly suggestive of a pregnant cow.

With the development of the fetus and growth of the feto-placental unit, there must be a corresponding increase in blood delivery to the uterus. This requires considerable enlargement of the uterine arteries, and by four to five months of gestation not only can the increased diameter of the artery supplying the gravid horn be appreciated but fremitus (a buzzing or vibrating sensation) as well. The uterine arteries originate from the aorta just caudal to the internal iliac artery and are freely movable as they traverse the broad ligament, allowing them to be readily differentiated from the internal iliac arteries (see Figure 22.11).

Misdiagnosis and Differential Diagnoses

Failure to properly determine the pregnancy status of a cow is most commonly a result of an incomplete examination and inexperience. This can be exacerbated by the desire for speed and fatigue. Therefore it is important – especially when first learning to diagnose pregnancy – to develop a systematic routine for the examination to be followed at all times and to allot adequate time for the procedure, with additional time for rest if needed to prevent fatigue from becoming a deleterious factor. It should be reiterated that in the absence of one of the four cardinal signs of pregnancy, a definitive diagnosis should not be made and the temptation to base a decision on supportive signs of pregnancy must be resisted. Indeed, these signs are not considered definitive indications of pregnancy because they may be induced by other conditions. Adhesions from a number of disease processes may fix the cervix and uterus in location. Fluid in the uterus may be the result of a pyometra or mucometra, rather than fetal fluid. Along with causing fluctuance of the uterus, this fluid may make the cervix and uterus difficult to retract in addition to causing asymmetry and/or enlargement of the uterine horns. Physometra (air within the uterus) may also cause asymmetry and enlargement of the uterus. Typically, however, in these cases the uterine walls do not thin as they would in pregnancy, yielding somewhat more tone to the feel of the uterus. Tumors may also make the cervix difficult to retract and cause asymmetry of the horns. These pathological conditions may cause enlargement and – albeit rarely – fremitus of the middle uterine arteries. Fremitus and uterine artery enlargement may also persist for a short time following delivery of a calf.

The uterus is a site of predilection for tumors caused by bovine leukemia virus, and in addition to altering the

supporting signs of pregnancy, these and other tumors may be difficult to differentiate from a fetus. With careful palpation it can be noted that this mass has no identifiable fetal parts such as the head or legs and is not floating within the normal fetal fluids but is in intimate contact with the endometrium. Similarly, a mummified or macerated fetus can be palpated as a mass within the uterus, and, although fetal parts may be identified, once again there is no amniotic or allantoic fluid surrounding the fetus. The uterine walls are contracted down around the remnant of the fetus and it is not freely movable with the uterine lumen. In addition, a macerated fetus often has a characteristic grinding or crepitus upon palpation, believed to be the body parts and bones rubbing against one another during manipulation. Cows having an enlarged cervix may occasionally be misdiagnosed as pregnant by the inexperienced examiner as the cervix may approach softball size and be very firm, feeling a bit like the fetal head or pelvis. The postpartum uterus is also enlarged, may be asymmetric, and may lead one to suspect pregnancy. However, the uterine walls will be very thick, the cardinal signs cannot be identified, and often rugae (ridges or folds of the surface of the uterus as it begins to shrink and involute following parturition) may be palpated.

Various structures not associated with the uterus may also occasionally be mistaken for pregnancy. The dorsal sac of the rumen can extend near or into the pelvic canal, particularly in a cow restrained in a hydraulic squeeze chute, and is sometimes mistaken for a large fetus by the beginner. It should be noted that it is much more "doughy" than the fetus. The right kidney may be bumped and may feel somewhat similar to ballotting a fetus; however, it is located more dorsally than a calf would normally be. The bladder is also sometimes mistaken for a fluid-filled uterus and must be differentiated from a pregnancy. Finally, tumors or other masses may be misdiagnosed as a fetus. With care and patience, a thorough examination of all of these will allow the clinician to determine that these are not associated with the reproductive tract and that the cardinal signs of pregnancy are not associated with these structures.

Determining the Stage of Gestation

Once a cow has been determined to be pregnant, both the cardinal and supportive signs of pregnancy can be utilized to make a determination as to the stage of her gestation. This determination can be very accurate (within a few days) during the first trimester of pregnancy, as fetal development through this time is relatively consistent regardless of breed or other genetic and environmental factors. Fetal development begins to vary a bit among individuals through mid-gestation and relatively more during the final trimester. Indeed, in the final trimester as the fetus goes through rapid growth, the examiner often has no precise way of predicting the size of the calf at birth. As birth weight may vary from less than 25 kg to more than 50 kg, depending upon breed and maternal and paternal factors as well as environmental factors, estimation of gestation length at this time becomes somewhat less precise than earlier in gestation. However, if the cattle are from a purebred operation or dairy farm, the examiner can have a reliable frame of reference from which to work. In a commercial herd, the size and type of the dam and any information about the type and breed of bulls being used will be helpful in achieving the highest degree of accuracy possible. The fingers and hand can be measured and used to evaluate the size of the structures being examined to further enhance the degree of accuracy.

Early in gestation, the size of the amniotic vesicle, the magnitude of the membrane slip, and the diameter of the horn can be used to determine the stage of gestation. As the pregnancy approaches 60 days and the fetus becomes palpable, it becomes another tool that can be used along with horn diameter. The size (Table 38.2) of either the fetus (Figure 38.2) or the nose to crown length of the head (Figure 38.3) may also be utilized.

By 4.5–5 months of pregnancy, the uterus is pulled deep into the abdomen of the cow and the fetus itself often may not be palpated. The development of placentomes by around day 75–80 provides another aid. While the size of the placentomes is very useful, just their presence combined with the inability to reach the fetus places the cow in mid-gestation. Placentome size is most consistent just anterior to the cervix. The diameter of the uterine artery(ies) may also be measured reliably at this time and the advent of fremitus ipsilateral to the pregnant horn indicates a pregnancy of about four months. Bilateral fremitus is usually present by about six months of pregnancy. As the fetus reaches seven months of age, it will begin ascending once again, allowing feet, legs, and head to be examined. It is important to note that at this time the placentomes become more variable in size and less reliable indicators of gestation length. See Table 38.2 for a list of pertinent structures and where they may be located at different stages of gestation as well as their approximate sizes as pregnancy develops.

Risks Associated with Transrectal Palpation

Although palpation per rectum is a relatively non-invasive procedure, it is not without some concerns. These include the possibility of disease transmission and causing damage

Table 38.2 Size of various reproductive organs and fetal structures that can be used to help determine the stage of gestation.

Gestation length (days)	Amniotic vesicle size (cm)	Membrane slip	Fetal body length (cm)	Fetal size	Placentome size	Fetal nose to crown length	Horn diameter	Uterine artery findings and diameter
30	0.6–0.7	Thread in gravid horn					Slightly asymmetric	3–4 mm
40	1.2–1.5	Small string in gravid horn					Slightly enlarged, asymmetric	
50	5–6						6 cm	
60	8–8.5	String in both horns	5.5–7	Mouse			7 cm	3–4 mm
70			10			1.5 cm		
80			14		Pea	3.5 cm		
90		Large string in both horns	15–16	Rat	Dime	5.5 cm	8–10 cm	
100			18–20			7.5 cm		
110			24–25			9 cm		
120			27–28	Small cat	Nickel–quarter	10.5 cm	15 cm	Fremitis on side of pregnant horn, 6 mm
150			36–38	Big cat	Half dollar		18 cm	Fremitis on side of pregnant horn, 9 mm
180			47–50	Beagle	Variable			Fremitus both horns, 10–12 mm
210			62–64	Springer Spaniel				
240			73–80	Doberman				
270			85–105	German Shepherd				10–15 mm



Figure 38.2 Two- to six-month-aged fetuses. *Source:* Courtesy Maarten Drost, VISGAR.



Figure 38.3 Palpation of fetal head. *Source:* Courtesy Maarten Drost, VISGAR.

to the embryo or fetus especially during early gestation. The diseases most frequently mentioned are bovine leukosis virus (BLV) and bovine virus diarrhea (BVD). Studies of both have met with mixed results. BLV has been shown experimentally to be spread when blood from infected cattle was repeatedly introduced rectally to non-infected cattle [1]. However, the same researchers in another study showed no transmission of the virus in a commercial dairy when the same glove was used on multiple cows versus using a new glove for every cow. In this same report, they demonstrated transmission of BLV in a university-owned teaching herd apparently from the use of common gloves. In this instance, it seems likely that more blood and trauma from inexperienced palpators and repeated palpations contributed to the seroconversion of these cows [2]. Conversely, in a small study, Kohara et al. [3] demonstrated seroconversion in three of four steers palpated a single time with a common glove following palpation of a positive cow. In addition, BLV negative cows palpated twice weekly for nine weeks with obstetric sleeves which were first used for aggressive palpation (to the point there was mucus and blood on the sleeves) of two BLV positive cows resulted in all the negative cows developing BLV antibodies [4]. Thus, it may be prudent to change or wash a sleeve if there is blood on the palpation sleeve prior to examining the subsequent cow in herds in which BLV is a concern.

Lang-Ree et al. [5] tied 10 BVD negative heifers side by side with no partition between them. They then palpated a persistently infected (PI) heifer and using the same sleeve palpated the first three heifers in the line. With a new sleeve, they again palpated a PI heifer and examined heifers 5–7. Again changing to a new sleeve, heifers 9 and 10 were palpated following the same procedure. All eight of the palpated heifers showed mild clinical signs of BVD and seroconverted within two weeks of the examinations. In addition, the virus was isolated from five of the eight palpated heifers. Blood was not observed at any time on the sleeve following palpation of the PI heifer. Heifers 4 and 8 (not palpated) remained asymptomatic and seronegative even though they were kept in close proximity with the others [5]. Consequently, in herds to be examined for pregnancy which are at high risk for having a PI cow, the clinician may want to take extra precautions to reduce the chance of transmitting the virus and inducing clinical signs or, more importantly, a PI calf (editor's note: see Chapter 62 for management of PI-BVD animals).

Early embryonic death resulting from transrectal palpation has also been studied with variable results. Older studies seemed to indicate some level of iatrogenic abortion during early pregnancy diagnosis. Females palpated at or before 45 days of gestation had higher incidence of embryonic mortality than females examined later in gestation [6]. Similarly, fetal loss was greater in females palpated 35–51 days after insemination than in females palpated 52–70 days post-insemination [7]. Decreased pregnancy rates were observed in females palpated twice between 42 and 46 days post-breeding as compared to females initially diagnosed pregnant by progesterone testing and first

palpated at 90 days post-breeding [8]. Vaillancourt et al. [9], on the other hand, found that though the rate of embryonic death was greater in females examined before 50 days of gestation, there was no conclusive indication that the increased embryonic loss was due to pregnancy diagnosis by fetal membrane slip. Conversely, Thurmond and Picanso [10] found that females palpated early in the gestational period from 28 to 42 days had a significantly lower probability ($P < 0.0001$) of early embryonic death compared with females palpated later in the 28–42 days interval. In two other studies, there was no significant difference ($P > 0.05$) in the incidence of embryonic death in females that underwent transrectal palpation and those that were not palpated [11, 12]. In a series of studies, Romano and colleagues demonstrated no deleterious effects of pregnancy diagnosis by membrane slip [13] or by palpation of the vesicle [14] versus ultrasound controls; and then in a comparison study on pregnancy loss, calving rates, and abnormalities in newborn calves, neither a difference between the two methods of diagnosis nor the control group which was diagnosed by transrectal ultrasound was identified [15]. A number of confounding factors have likely contributed to the differences in these studies. These include natural embryonic and fetal loss and the lack of a reliable, non-invasive test with which to make the original pregnancy diagnosis. As these tests have improved, the degree of normal embryonic and fetal loss has become better understood, thus allowing for better design and evaluation of the more recent studies. In addition, one must take into consideration the number of examinations and stress placed on cattle in studies compared with normal production scenarios. As these factors may contribute to the varying results of these studies [13, 16], it is likely that the more recent studies are more reliable and that routine transrectal palpation does not significantly contribute to early embryonic death or fetal demise when performed within normal management programs.

Summary

The cost of carrying open cows is significant to the beef producer, and for dairy producers it is important to minimize the calving interval for each cow to maximize profitability. Rectal palpation is currently the most commonly utilized method of diagnosing pregnancy in cattle. With experience it can be performed rapidly, with very good accuracy and relatively inexpensively, to determine not only pregnancy status but also stage of gestation. Indeed, Kasimanickam et al. [17] showed a very small error rate when trans-rectal palpation was used to separate cows that were 70 days pregnant by fixed time artificial insemination (FTAI) from cows that were bred by a bull turned in two weeks following the FTAI, and demonstrated increasing accuracy with the experience of the individual performing the examination. This kind of capability allows the producer the opportunity to make crucial management decisions such as culling or knowing when to dry a cow off based upon these findings. However, data gathered by the National Animal Health Monitoring System indicate that pregnancy diagnosis by palpation was utilized in only 10.8% of beef cattle operations with less than 50 cows, 25.8% of operations with 50–99 head, 41.2% of operations with 100–199 head, and 58.3% of farms with 200 or more cows [18]. On the other hand, about two-thirds of all dairy operations (67.0%) performed pregnancy examinations monthly or more frequently. The majority of large operations (75.0%) performed pregnancy examinations weekly or every two weeks, while 50.2% of small operations performed examinations on a monthly basis and 69.3% of mid-size operations performed examinations once or twice a month [19]. Thus, there is a significant opportunity for veterinarians with expertise in pregnancy diagnosis and pregnancy staging by rectal palpation to provide a valuable profit-building service to their clients and the effort to develop this skill is well worth the investment.

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39

Examination for Pregnancy: Biochemical Tests

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Introduction

Prompt accurate diagnosis of pregnancy or non-pregnancy is critical for efficient livestock management, especially in the dairy industry where profitability relies largely on the reproductive efficiency of the cow herd [1]. Early identification of non-pregnant cows, particularly if they can be identified before the next expected estrus, is vital for maintenance of a desirable calving interval in both beef and dairy herds.

Traditionally, producers have relied heavily on transrectal palpation and/or transrectal ultrasound to diagnose pregnancy. Both of these methods require specialized training and technical skill and are usually performed by a veterinarian. Neither can diagnose pregnancy prior to the estrus immediately following breeding. Moreover, many geographical areas are experiencing a shortage of large animal practitioners. This, coupled with the desire to identify non-pregnant animals as early after insemination as possible, has made the pursuit of reliable biochemical methods of pregnancy testing more attractive [2]. A number of biochemical tests for pregnancy in the bovine are currently available. These tests detect pregnancy-associated hormones or the presence of pregnancy-specific molecules in maternal circulation and are marketed as viable alternatives to transrectal palpation and transrectal ultrasonography. However, unlike transrectal palpation and transrectal ultrasound these tests by themselves cannot currently be used to closely estimate the stage of gestation.

When diagnosing pregnancy via transrectal palpation, reproductive structures are felt for positive signs of pregnancy. Most large-animal veterinarians can accurately diagnose pregnancy as early as 30–35 days after insemination, but the level of accuracy is highly dependent on the expertise of the veterinarian and generally increases when examination is delayed to 40–50 days after breeding [3]. Concerns over early embryonic mortality as a result of transrectal palpation, particularly via fetal membrane slip, have been expressed and have been the subject of multiple studies. The risks associated with transrectal palpation for diagnosis of pregnancy are fully addressed in Chapter 38.

Through transrectal ultrasound, pregnancy can be consistently and accurately diagnosed as early as 25 days after insemination. Maximum sensitivity in diagnosing a pregnancy is reached at day 26 and day 29 in heifers and cows, respectively [4, 5]. For this reason, transrectal ultrasound is often used as the "gold standard" when evaluating other means of pregnancy diagnosis. Although 25 days after insemination provides a diagnosis earlier than transrectal palpation, transrectal ultrasound still does not allow diagnosis of non-pregnancy before the next expected estrus, and, like transrectal palpation, requires that the veterinarian has a great deal of technical skill in order to yield consistently accurate diagnoses. Furthermore, this method of pregnancy determination requires substantial inputs to purchase and maintain necessary equipment, and these costs must be passed on to the producer.

The ideal pregnancy test would accurately diagnose both pregnancy and non-pregnancy a short time after fertilization, and have excellent sensitivity (the female that tests pregnant is indeed pregnant), specificity (the female that tests non-pregnant is indeed open), and accuracy (the proportion of true results). Furthermore, the ideal pregnancy test would be non-invasive in that there would be no risk of causing embryonic or fetal death through testing. In addition, it would be minimally expensive, require little or no technical skill, and could be quickly and easily performed chute-side. An ideal pregnancy test for the bovine would be very similar to the human chorionic gonadotropin (hCG) test for pregnancy in women.

The hCG test is a simple enzyme-linked immunosorbent assay (ELISA) that detects the presence of hCG protein in

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

the urine of pregnant women. It can easily and inexpensively be performed in the home, requiring virtually no technical skill. Commercially available hCG tests boast extremely high accuracy (>99%), and some can be performed as early as 8–10 days after conception [6]. Unfortunately, cows and heifers do not produce hCG or any analogous molecule that could be tested in a comparable fashion. Instead, tests have been developed to ascertain the presence of certain pregnancy-associated molecules such as elevated progesterone concentration, pregnancy-associated glycoproteins (PAGs), early pregnancy factor (EPF), and interferon-stimulated genes (ISG) [2]. Additionally, as technology and scientific knowledge of early pregnancy advance, there are a number of other molecules that may one day be used as pregnancy biomarkers in the bovine.

An important distinction that producers should be mindful of when opting to utilize biochemical methods to determine pregnancy status is that progesterone, PAG, EPF, and ISG assays are more accurate in detecting non-pregnant females than in correctly identifying pregnancy. Consequently, endocrine-based pregnancy tests often have excellent specificity (>90%) but slightly less desirable sensitivity. This is of significant economic importance because females in intensive production scenarios that are falsely identified as open will often be administered prostaglandin in an attempt to initiate a new cycle and reduce time to next insemination. This injection of prostaglandin will, of course, induce abortion in pregnant females, and since the average cost of a pregnancy loss in a dairy female in the USA is estimated at $555, there is significant incentive to minimize the incidence of iatrogenic embryonic and fetal loss [7].

Similarly, another confounding factor when attempting to identify pregnancy soon after fertilization is natural pregnancy loss. Bovine fertilization rates are usually high (>90%) and therefore the main contributor to reproductive wastage is embryonic or fetal death [8, 9]. Early embryonic death, occurring prior to day 17 of pregnancy, accounts for 20.5–43.6% of loss when coupled with frequency of failure of fertilization [10]. However, late embryonic death, which represents loss from approximately 17 to 42 days of gestation, is estimated to be, on average, 12.8% in dairy cows and approximately 6.5% in beef females [11–13]. It has been estimated that pregnancy losses from fertilization to term in high-producing dairy cows may approach 60% [13]. When critically evaluating the reliability of endocrine tests performed early in gestation, it is customary to confirm pregnancy diagnosis by transrectal palpation or ultrasound days or weeks after the initial test was performed. This creates a window in which embryonic or fetal loss can occur, thus skewing the data by incorrectly labeling some results as false positives as the female was indeed pregnant when first tested but lost the pregnancy before the confirmatory pregnancy evaluation.

Progesterone Concentration

Serum and milk progesterone levels steadily increase following ovulation as a functional corpus luteum (CL) grows and develops. In non-pregnant females, progesterone declines as the CL regresses and the cow returns to estrus. In pregnant females, however, progesterone levels should remain elevated during the period of the next expected estrus as the CL continues to produce progesterone in order to maintain the pregnancy.

Progesterone concentration testing functions on the premise that serum and/or milk progesterone concentration will remain elevated at the time of the next expected estrus if the female were pregnant. If the female is open, progesterone concentration will decrease to baseline as the CL regresses and a new dominant follicle develops [3] (see Chapters 24 and 25).

Discrepancies in progesterone testing may be largely caused by natural variation between individuals in luteal lifespan and estrous cycle length as progesterone levels remain high while a CL is present during diestrus [14]. Furthermore, embryonic mortality between the time of progesterone testing and pregnancy confirmation via transrectal palpation or transrectal ultrasound examination may contribute to perceived test error [3].

A potential drawback to the use of progesterone testing in production settings is that the breeding or insemination date must be known in order to obtain reliable results from a single sample, and therefore the applicability of progesterone testing is somewhat limited [2, 3]. Typically, a milk or serum sample is taken 21–24 days after breeding and is analyzed using either radioimmunoassay (RIA), ELISA, or, less frequently, latex agglutination (LA). A number of progesterone ELISAs for serum and milk are available to producers and are marketed as cow-side tests.

Progesterone testing is very reliable for identifying non-pregnant females, as cows with blood progesterone levels less than 1 ng/ml 21 days after insemination cannot be pregnant, but it is less reliable for positively diagnosing pregnancy because of natural variations in estrous cycle lengths between individual animals [2]. Detection rates of 95–100% have been reported in detecting non-pregnancy. Pregnancy was correctly identified 68–97% of the time, with study designs varying in the day of gestation the test was performed, the standard it was compared against, and the type of assay used (ELISA, RIA, or LA) [15–20].

Pregnancy-Associated Glycoproteins

Ruminant trophoblasts release proteins as part of maternal recognition of a viable conceptus. These pregnancy-associated glycoproteins (PAGs) are aspartic peptidases and have not

been found to be proteolytically active. The first PAG to be discovered was pregnancy-specific protein B (PSPB), formerly known as PAG 1. This protein is produced by trophoblast binucleate cells in the bovine placenta around the time of implantation, though its exact role in pregnancy maintenance is not yet fully understood. It can be found in maternal circulation from the time of implantation throughout gestation, often persisting well into the postpartum interval [21, 22].

As its name suggests, PSPB is a pregnancy-specific molecule and is not found in the serum of virgin heifers or open cows, thus demonstrating that the protein is produced solely as a response to a conceptus and not in response to luteal activity during the estrous cycle [23]. Unlike equine chorionic gonadotropin (eCG) and hCG, PSPB is not detectable in urine, and it is only found in milk during intervals when PSPB is at very high concentration in plasma, for example shortly after parturition [21]. For additional information regarding the origins and characterization of PAGs, specifically PSPB, see the review article by Wallace et al. [24].

An RIA capable of reliably detecting PSPB in maternal serum as early as 24 days after conception was developed by Sasser et al. [23] in 1986. A then-unique advantage of PSPB testing was that, unlike progesterone assays, insemination dates are not needed to confirm pregnancy in PSPB testing as the protein is pregnancy specific and present throughout gestation, peaking around the time of parturition [23].

In addition to the RIA test, there are several commercially available ELISAs for bovine PAGs including PSPB. Currently available tests include BioPRYN™ (Biotracking, Moscow, ID, USA), Bovine Pregnancy Assays (IDEXX Laboratories Inc., Westbrook, ME, USA), and DG29 (Conception Inc., Beaumont, Quebec, Canada). These assays are marketed in both the USA and abroad as convenient and inexpensive pregnancy tests for use by both producers and veterinarians, and furthermore are marketed as being as accurate as transrectal ultrasound examination. They can be used as early as 28 days after insemination in both cows and heifers and use similar concentrations for the determination of pregnancy or non-pregnancy as earlier RIA tests [24–26]. According to the manufacturers, samples (blood or milk) may be submitted to labs and results will be reported to producers the same day that samples are received, or kits are available that allow the assay to be run on farm in as little as 30 minutes. When verified contemporaneously with transrectal ultrasound near the end of the late embryonic period, PAG ELISAs yielded accuracies for pregnancy detection ranging from 89 to 100% [27–31], and on-farm rapid visual test kits were found to have equal sensitivity and accuracy as traditional PAG ELISAs [32].

The usefulness of PAGs in pregnancy diagnosis can be detrimentally affected by the relatively long half-life of some PAGs. For example, PSPB can be detected in maternal blood up to 80–100 days after calving; therefore producers must be cautious when using PSPB assays to detect pregnancy in cows bred early in the postpartum period [23, 33]. In addition, this long half-life may result in false-positive tests for pregnancy following pregnancy loss. Finally, this test does not have the advantage of diagnosing non-pregnancy prior to the cow's first estrus following breeding.

Early Pregnancy Factor

EPF is an immunosuppressive hormone that can first be found at detectable levels in the maternal system 24–48 hours after successful fertilization and persists for at least the first half of gestation [34]. EPF acts as an immunomodulator protecting the antigenically foreign conceptus from maternal rejection [35]. It is produced by maternal tissues during the preimplantation stage in response to the presence of a conceptus. After implantation has occurred, however, the embryonic tissue assumes responsibility for EPF production. Only a viable conceptus that has reached the blastocyst stage is capable of producing EPF. Furthermore, EPF dissipates rapidly after the death or removal of the conceptus. Unlike some PAGs, including PSPB, EPF has never been detected after parturition [34]. The combination of these attributes makes EPF potentially very useful for pregnancy determination and recognition of embryonic fetal death.

Before the discovery of EPF and development of a test to determine its presence, it was impossible to detect a pregnancy prior to implantation except through invasive surgery. Although diagnosing pregnancy so soon after insemination seems appealing, the high frequency of early embryonic mortality makes this test less useful. Producers opting to use EPF to diagnose pregnancy early in gestation should retest all positive females at a later date to confirm the presence of a viable conceptus.

A producer-oriented assay for EPF would be a very valuable tool for the detection of fertilization and pregnancy in cows during the first four weeks of gestation as the unique properties of EPF make it particularly useful in intensive production scenarios. It is detectable within 48 hours of fertilization and, unlike PAGs, disappears rapidly on removal of the embryo or fetus, and therefore can be assayed accurately even in cows inseminated early in the postpartum interval that may have residual PAGs in circulation. Furthermore, an EPF assay would be far less likely than a PAG assay to yield a false-positive result due to previous pregnancy loss [36]. Most importantly, however, an EPF test can accurately determine the pregnancy status of a female well before her next expected estrus [34].

The first and currently the only reliable test for EPF is the rosette inhibition test (RIT) in which EPF, in the presence of a complement, works to cause an increase in the effects and titers of antilymphocyte serum in decreasing the

formation of rosettes between lymphocytes and heterologous red blood cells [34]. The RIT for EPF is very accurate and extremely useful in identifying non-pregnant females. Unfortunately, the RIT is time-consuming and difficult to maintain, and therefore it is not practical for regular use on commercial operations [3].

A producer-oriented test to detect the presence of EPF was previously available in the USA. This test, the early conception factor (ECF) lateral flow assay, was marketed as a cow-side test for non-pregnancy and reportedly could detect EPF in milk or serum 48 hours to 20 days after fertilization [37]. However, concerns regarding the accuracy of the ECF test performed early in gestation were raised, and the ECF test is no longer commercially available. Ambrose et al. [38] reported that ECF specificity on 7, 14, and 21 days after insemination ranged from 38 to 51%. Data from other studies corroborate these findings and conclude that the ECF lateral flow assay cannot correctly identify open cows with the degree of precision needed by most commercial dairy operations [39–41]. However, the development of a more reliable assay for EPF could revolutionize early bovine pregnancy diagnosis, especially in the dairy industry.

Interferon-Stimulated Genes

In cattle, the molecule primarily responsible for the maternal recognition of pregnancy is interferon (IFN)-tau. It is secreted by conceptus trophectoderm cells and acts in a paracrine manner on uterine endometrium to inhibit pulsatile secretion of prostaglandin (PG)$F_{2\alpha}$, thereby effectively inhibiting luteolysis and successfully maintaining the pregnancy [42]. Unlike the molecules of maternal pregnancy recognition in some other mammalian species (i.e. hCG in humans and eCG in mares), IFN-tau is present only in very low concentrations in the maternal periphery and therefore cannot currently be directly tested for in serum or milk. Thus, maternal IFN-tau concentration itself cannot be used in diagnosing early pregnancy in the bovine [43].

In addition to its role in interrupting the signal transduction pathway that regulates the release of $PGF_{2\alpha}$, IFN-tau induces the upregulation of multiple differentially expressed ISGs on maternal leukocytes. Among these ISGs are myxovirus resistance gene 1 and 2 (Mx1 and Mx2) and interferon-stimulated gene 15-kDa protein (ISG15), a ubiquitin homolog [43–46]. ISG15 mRNA expression on maternal white blood cells follows a similar pattern to IFN-tau release, first being expressed around day 14 after conception, peaking between day 17 and 18, and declining from day 21 to day 26 [47]. However, unlike IFN-tau, ISG15 is detectable in the maternal periphery in early pregnancy and could potentially be useful in diagnosing non-pregnancy before the next expected return to estrus. Because of variability between individual females' white blood cell composition and differences in IFN-tau concentrations between conceptuses, ISG15, like most other molecules of biochemical pregnancy diagnosis, is more useful in detecting open cows than in affirmatively diagnosing pregnancy.

Though results generated in laboratories for the use of ISG15 in identifying non-pregnant females, especially heifers, have been promising, thus far a consumer-oriented test for ISG15 mRNA upregulation has not been developed, and real-time polymerase chain reaction (PCR) must be used to detect ISG15 mRNA in maternal serum [43, 48]. Furthermore, serial blood tests are necessary to achieve maximum accuracy (>99.9%) when using ISG15 mRNA to identify non-pregnant cows and heifers. Accuracy in detecting non-pregnant cows from a single blood collection (day 18) was 89% in a 2006 study conducted by Han et al. [48]. Further complicating the potential application of ISG15 as a means to early pregnancy determination is ISG15 activation as a response to interferon throughout the course of a viral infection. If relying solely on ISG mRNA expression levels for pregnancy detection, virally infected open females may be falsely diagnosed pregnant [48]. Though ISG15 mRNA is currently not feasible for use in early pregnancy determination in commercial operations, with refinement and further scientific advances this innovative new technology may one day be adapted for widespread use in diagnosing pregnancy and predicting embryonic mortality.

Emerging Research

In addition to the previously described pregnancy-associated molecules, which have been well tested and reviewed in scientific literature, there are a number of other peripheral indicators of pregnancy that may one day be useful for commercial pregnancy diagnosis in the bovine. As our knowledge of early gestation and early maternal responses to conception increases, more biomolecules with potential applications in pregnancy determination will be uncovered. As with existing biochemical pregnancy tests, these new and emerging tests will endeavor to detect the presence of a conceptus prior to the female's next expected estrus so that she may be rebred, if necessary, as quickly as possible.

Among the most promising molecules for potential future use in early bovine pregnancy determination are circulating nucleic acids (CNAs). CNAs are cell-free DNA/RNA molecules circulating in the blood and have been used as biomarkers in both diseased and healthy humans and animals for a number of years. In humans, CNAs are primarily used in the diagnosis of certain tumor diseases and other disorders. During human pregnancy, CNAs are often used to screen for genetic abnormalities such as chromosomal aneuploidy [49]. Unlike human fetuses, bovine

Table 39.1 Summary of biochemical indicators presently used for the diagnosis of pregnancy in the bovine.

Molecule	Type of test	Producer test available?	Days of gestation effective	Significant drawbacks
Progesterone	RIA, ELISA, LA	Yes	21–285	Must know breeding date to use reliably in early gestation
PAGs	RIA, ELISA	Yes	28–285	Long half-life may skew results in females bred before 90 days postpartum
EPF	RIT	No	2–140	No producer test
ISG15	RT-PCR	No	14–26	No reliable producer test; virally infected females may have false positives

trophoblasts have no direct contact with maternal blood, and therefore exchange of fetal genetic material occurs less readily than it does in humans. However, it has been demonstrated that "transplacental leakage" of bovine fetal DNA into the maternal circulation can, and often does, occur in spite of the epitheliochorial placenta structure, though the mechanisms responsible for this exchange have not yet been elucidated [50, 51]. Other studies have demonstrated that CNAs can be used to accurately (100% for male calves, 91% for female calves) determine calf sex after day 30 of gestation [51, 52]. Mayer et al. [49, 53] have recently characterized the differences in CNA composition between pregnant and open cows at day 20 of gestation, finding multiple sequences present exclusively in the serum of pregnant females. Although this particular application of CNA technology is still in its infancy, these findings indicate that it may one day be feasible to use CNAs to identify pregnant cows and heifers as early as 20 days after breeding.

Another promising class of biomarkers for potential use in early pregnancy diagnosis is microRNA (miRNA). MiRNAs are short, non-coding RNA molecules which are heavily involved in gene expression and may be found in virtually every bodily fluid, making them ideal for minimally invasive sampling [53–56]. At this point in time, numerous miRNAs have been identified as unique to individual disease or physiological states, including pregnancy [56–58]. Further studies are needed to elucidate the role of miRNAs in bovine early pregnancy and to determine which miRNA molecules would be most reliable in the diagnosis of pregnancy [59].

Summary

Intensive reproductive management systems make early and accurate pregnancy determination a necessity in the cattle industry, especially on commercial dairy operations. Though producers have traditionally been limited to transrectal palpation and transrectal ultrasound for pregnancy diagnosis, a variety of biochemical pregnancy tests are rapidly becoming available. A summary of the tests discussed in this chapter and their attributes is provided in Table 39.1. All of these tests have the potential to diagnose pregnancy or non-pregnancy earlier than traditional methods (transrectal palpation and transrectal ultrasound) while still maintaining a comparable level of accuracy. However, on their own, they do not have the capability to closely stage the pregnancy and may require some knowledge of the breeding or artificial insemination (AI) date.

Currently, only EPF or ISG15 would allow producer intervention (i.e. prostaglandin administration) prior to the first estrus after insemination. Unfortunately, RIT and real-time PCR, respectively, are required to accurately detect these molecules in maternal peripheral circulation. Both of these tests require expensive equipment and are difficult to run outside of a laboratory environment. Thus, the practicality of using these compounds for bovine pregnancy determination is currently limited. Producers and veterinarians should work together to make decisions regarding which pregnancy testing method to use based on the individual needs of each operation.

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Ultrasound Evaluation of the Female Reproductive Tract

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Introduction

When O.J. Ginther [1] published *Ultrasonic Imaging and Animal Reproduction: Fundamentals* in 1995, field veterinarians began using ultrasound technology for reproductive examinations in the bovine. With the advent of small machines designed to be carried on the operator's person, the use of ultrasound has become mainstream. Many texts and other resources have been published describing the use of ultrasound for bovine reproductive examinations [1–6]. The goal of this chapter is not to reiterate those resources, but to expand on them.

Pregnancy Diagnosis

It is theoretically possible to identify a conceptus by ultrasound as early as 21 days after breeding [7], but that is usually not practical in field conditions. One proficient ultrasonographer tested his ability to accurately diagnose open versus pregnant cows beginning at 24 days after breeding [8]. The negative predictive value (diagnosis of not pregnant) was 89% at 24 days in adult cows. In other words, 11% of cows diagnosed open were found to be pregnant when rechecked at 32 days. By 26 days this ultrasonographer was able to identify open cows correctly in 99% of cows examined. In heifers he was able to achieve the same level of competency at 24 days after breeding.

Bovine ultrasonographers are advised to perform a similar analysis of their skills to identify their competence at various stages after insemination. The time it takes to perform examinations and the number of rechecks required at various stages should also be taken into consideration. Note the large difference in the amount of fluid and size of the embryo between 26 days (Figure 40.1a) and 28 days (Figure 40.1b). Note that the 26-day embryo is difficult to visualize against the uterine wall. The 28-day embryo, on the other hand, is 1 cm in length and easily seen within the uterine fluid. Embryonic development occurs very rapidly, so waiting just a couple of days can improve accuracy and speed of pregnancy examinations. It is of no value to the producer if early pregnancy examinations do not lead to immediate management decisions.

Ovarian structures are helpful aids in pregnancy diagnosis. It can hasten the examination to know if a corpus luteum (CL) is present and, if so, on which side. Two corpora lutea indicate the potential for twins. A pregnancy with the CL contralateral to the embryo or fetus is very high risk for pregnancy loss. A small or poor-quality CL may indicate low progesterone levels that could lead to pregnancy loss. A cavitary CL is of no concern unless the cavity is so large and the rim of luteal tissue so small that total luteal volume is low. In cases of poor-quality or contralateral corpora lutea, treatment with gonadotropin-releasing hormone (GnRH) may produce an accessory CL to improve progesterone levels. Examination of ovarian structures is discussed further in later sections of this chapter.

The producer should understand that early diagnosis of open cows is critical for quick rebreeding of those animals, but that pregnant cows diagnosed before day 60 should be checked again after placentation is complete (see section "Embryonic and Fetal Viability"). This is especially important for lactating dairy animals due to their higher risk of late embryonic and early fetal death.

Embryonic and Fetal Aging

Ultrasound is far superior to manual palpation for staging pregnancies up to about 120 days in gestation. With ultrasound, embryonic or fetal size is measured rather than the size of the amniotic sac or total amount of pregnancy fluid. Embryonic and fetal growth is very consistent

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

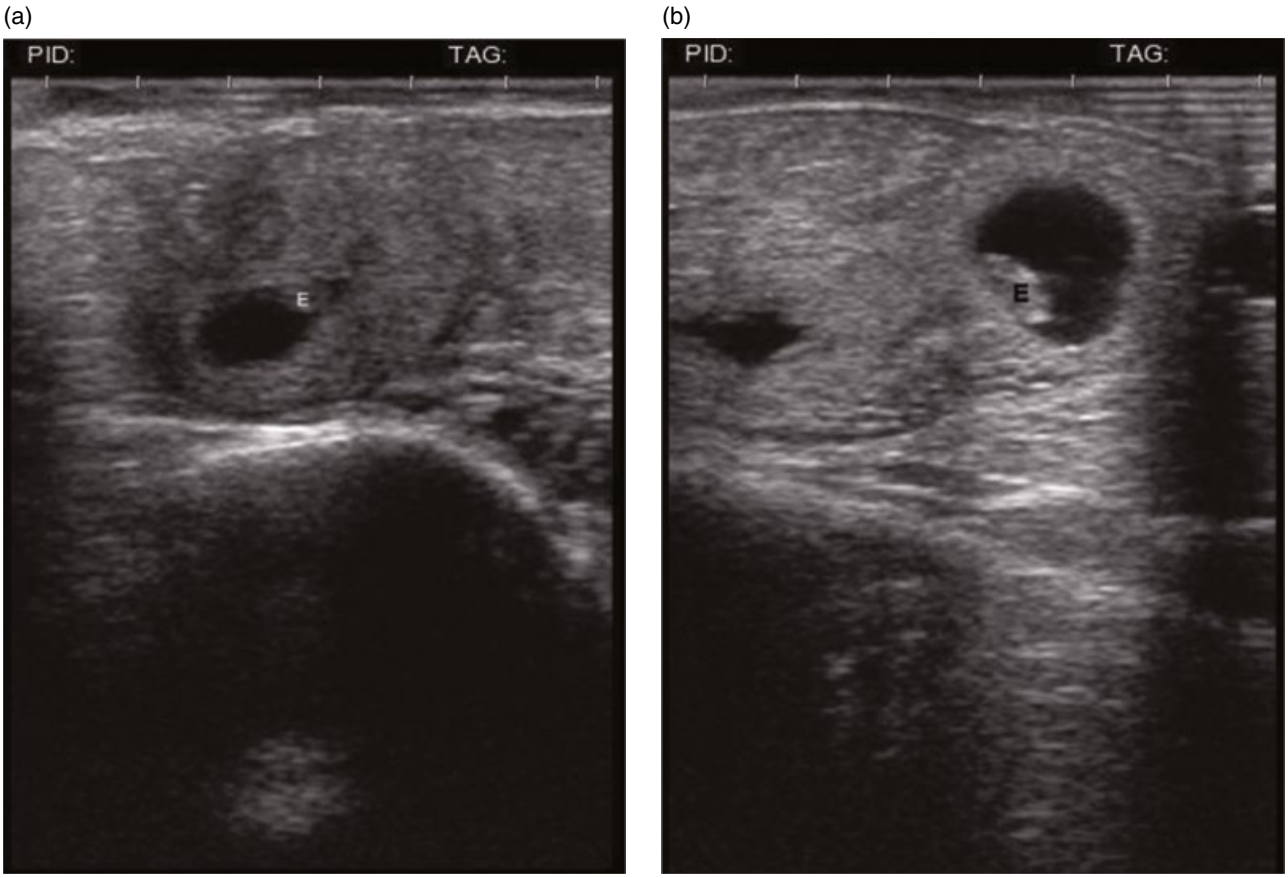


Figure 40.1 (a) A 26-day pregnancy and embryo (E). (b) A 28-day pregnancy and embryo (E).

Table 40.1 Ultrasonic appearance of embryonic and fetal structures by days in gestation.

Characteristic	Earliest mean	Range
Embryo proper	20.3	19–24
Heartbeat	20.9	19–24
Allantois	23.2	22–25
Spinal cord	29.1	26–33
Forelimb buds	29.1	28–31
Amnion	29.5	28–33
Eye orbit	30.2	29–33
Hindlimb buds	31.2	30–33
Placentomes	35.2	33–38
Split hooves	44.6	42–49
Fetal movement	44.8	42–50
Ribs	52.8	51–55

Source: Adapted from [7].

regardless of ambient temperature or breed, whereas the amount of fluid can vary.

Two methods can be used to determine the gestational age of the embryo or fetus. First, direct measurements of the embryo or fetal structures can be taken and correlated to age using a gestational aging chart [9, 10]. Trunk diameter is the widest part of the ribcage at the level of the umbilicus. Braincase diameter is the maximum diameter of the skull just caudal to the eyes. Head length is measured from the top of the cranium to the tip of the nose. Crown–rump length is measured from the tailhead to the crown of the skull. Of these measurements, only crown–rump length has a simple formula for a portion of gestation. Until approximately 50 days of gestation embryonic or fetal age can be calculated thus:

$$\text{Length of the embryo fetus in millimeters} + 18 = \text{embryonic or fetal age in days}$$

For measurements of other structures, or for fetal ages greater than 50 days, one must use fetal aging charts or an ultrasound unit that is equipped with software to measure and calculate gestational age.

The second method is to visually evaluate embryonic/fetal development. Sandra Curran and colleagues have studied when various structures first appear (Table 40.1) [7]. This author finds that in field conditions these structures are most easily seen at the high end of Dr. Curran's range. Using embryonic/fetal size and knowledge of development,

experienced ultrasonographers learn to "eyeball" age quite accurately without referring to charts or software.

After approximately 120 days of gestation fetal aging is less accurate. It is difficult to reach and measure fetal structures. Also, fetal size begins to vary among animals with the same breeding dates. Other measurements, such as fetal eye diameter, placentome size, or uterine artery diameter, are too variable among animals to provide accurate estimates of fetal age.

Embryonic and Fetal Viability

Lactating dairy cattle experience considerable pregnancy loss, particularly before 60 days of gestation when placentation is not yet complete. Losses in lactating dairy cattle are approximately 3.6 times greater than in pregnant heifers [11] and often are 10–12% from day of initial pregnancy diagnosis to 90 days [12]. Hypotheses for this high rate of loss include reduced progesterone levels due to high milk production [13], a history of postpartum disorders [14], insemination by certain bulls [15], twinning [16], and heat stress [17]. Regardless of cause, it is critical for producers to understand pregnancy loss and the need to reconfirm pregnancies diagnosed prior to about 60 days of gestation.

Fetal viability is easily assessed with ultrasound. Beginning at about 24 days of gestation, the fetal heartbeat can be seen [7]. The heart rate should be at least 130 bpm in early gestation, increasing to about 190 bpm by 60 days [2]. It can be difficult to identify the beating heart if the cow or the fetus is moving. For this reason, it is advisable to recheck pregnancies when no heartbeat is seen but all else appears normal.

The amniotic and chorioallantoic fluids should be clear and anechoic until at least 70 days of gestation. If the fluid is cloudy before 70 days and there is no heartbeat, the embryo or fetus can safely be assumed to be dead. Separated chorioallantoic membranes may also be seen in cases of late embryonic or early fetal death, often appearing to be floating in the pregnancy fluids. Compare the normal 35-day pregnancy in Figure 40.2a with the dead 40-day twin pregnancy in Figure 40.2b. Note the clear fluid and normal fetal development in the live fetus versus the flocculent fluid and unformed fetuses in the dead twins. Movement, other than a beating heart, is difficult to detect before 50 days because the limbs are not well developed yet.

After 70 days the fluids of pregnancy may begin to appear cloudy in normal pregnancies, but by that stage fetal movement can easily be appreciated. Separation of the chorioallantoic membrane is difficult to appreciate at this stage due

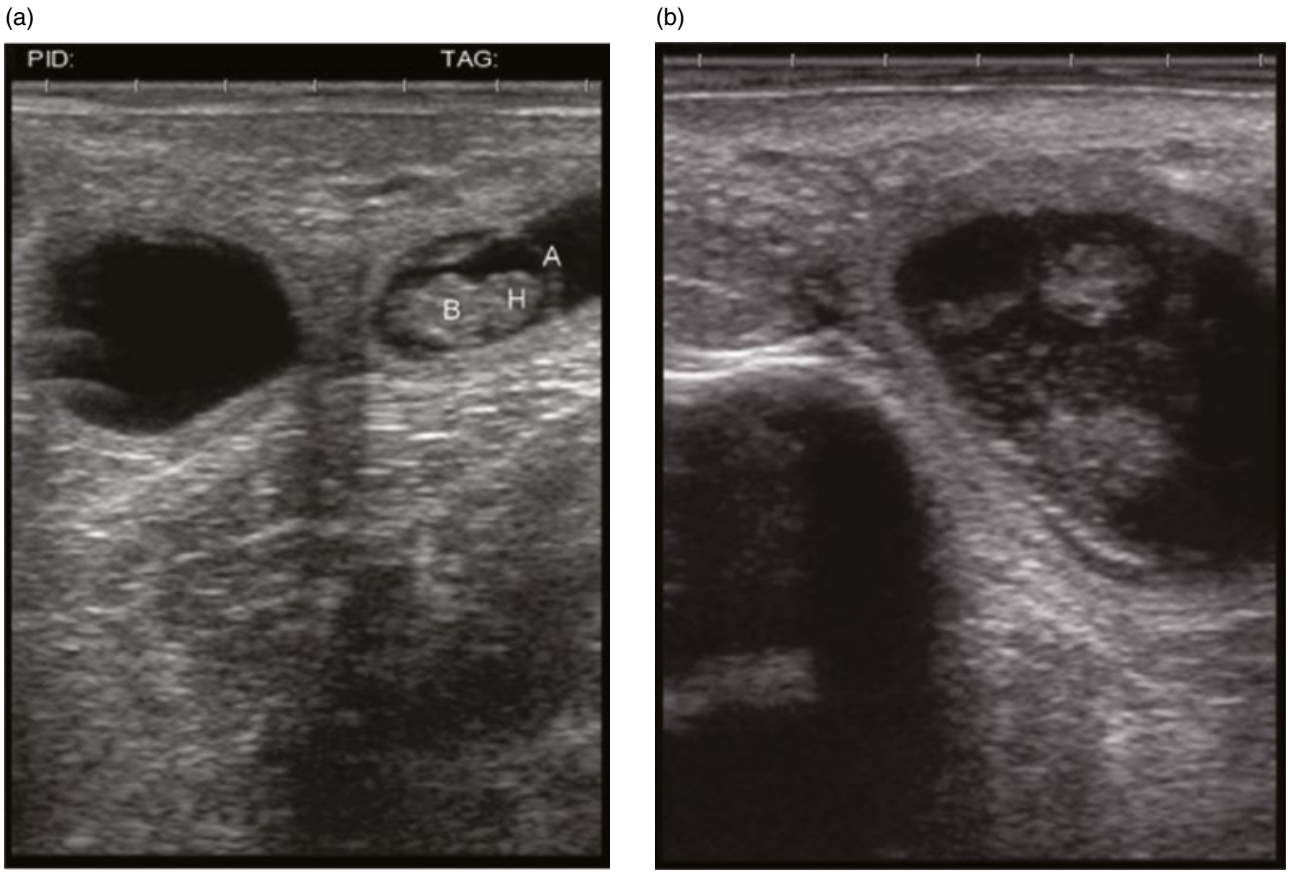


Figure 40.2 (a) Normal 35-day pregnancy: amniotic vesicle (A), head (H), body (B). (b) Dead 40-day twins.

to elongation and folding of all the placental tissues. Dead fetuses often are very heterogeneous, with many areas of high echogenicity that do not correspond to normal bony anatomy. There may also be fluid in the peritoneal cavity. Compare the dead 65-day fetus in Figure 40.3a with the normal 60-day fetus in Figure 40.3b. Fetal mummies (Figure 40.4) are easily identified by echogenic bony remnants, nearly complete lack of intrauterine fluid, lack of fetal movement, and lack of heartbeat.

In the absence of prostaglandin treatment and CL regression, dead embryos and fetuses can persist for weeks [18]. In these cases there are usually still palpable cardinal signs of pregnancy such as membrane slip or amniotic vesicle (AV). With ultrasound, however, they can be quickly identified without additional examinations and treated with prostaglandin. In most cases, expulsion of the dead embryo/fetus and uterine contents will be complete within 48 hours after prostaglandin administration.

In some cases, fetal distress can be identified before death. A heart rate of less than 130 bpm is cause for reevaluation at the next visit. Mild separation of chorioallantoic membranes or abnormal fluid in the presence of a live fetus is also cause for follow-up. A live fetus smaller than expected may be cause for concern, but is usually due to incorrect recording of breeding date. Twin fetuses are not smaller than singletons until late in gestation. Fetal anomalies are at higher risk of loss throughout gestation and, in many cases, it is desirable to abort these abnormal pregnancies intentionally (see section "Embryonic and Fetal Anomalies"). As discussed at the beginning of this section on "Pregnancy Diagnosis," a contralateral or poor quality CL is also reason to recheck the pregnancy at the next opportunity.

Twins

High twinning rates are often seen in dairy herds and, less commonly, in beef herds. The primary mechanisms for twinning appear to be correlated to high milk production in dairy cattle [19] and to genetic propensity in certain strains of beef cattle [20]. In dairy cattle there also appears to be a higher risk of twinning in cows with a history of cystic ovarian disease [16]. In any case, twinning can lead to more pregnancy loss [16, 21], more dystocia, more periparturient disease, reduced future reproductive performance [22], freemartinism, and a higher risk of early culling for the dam [23].

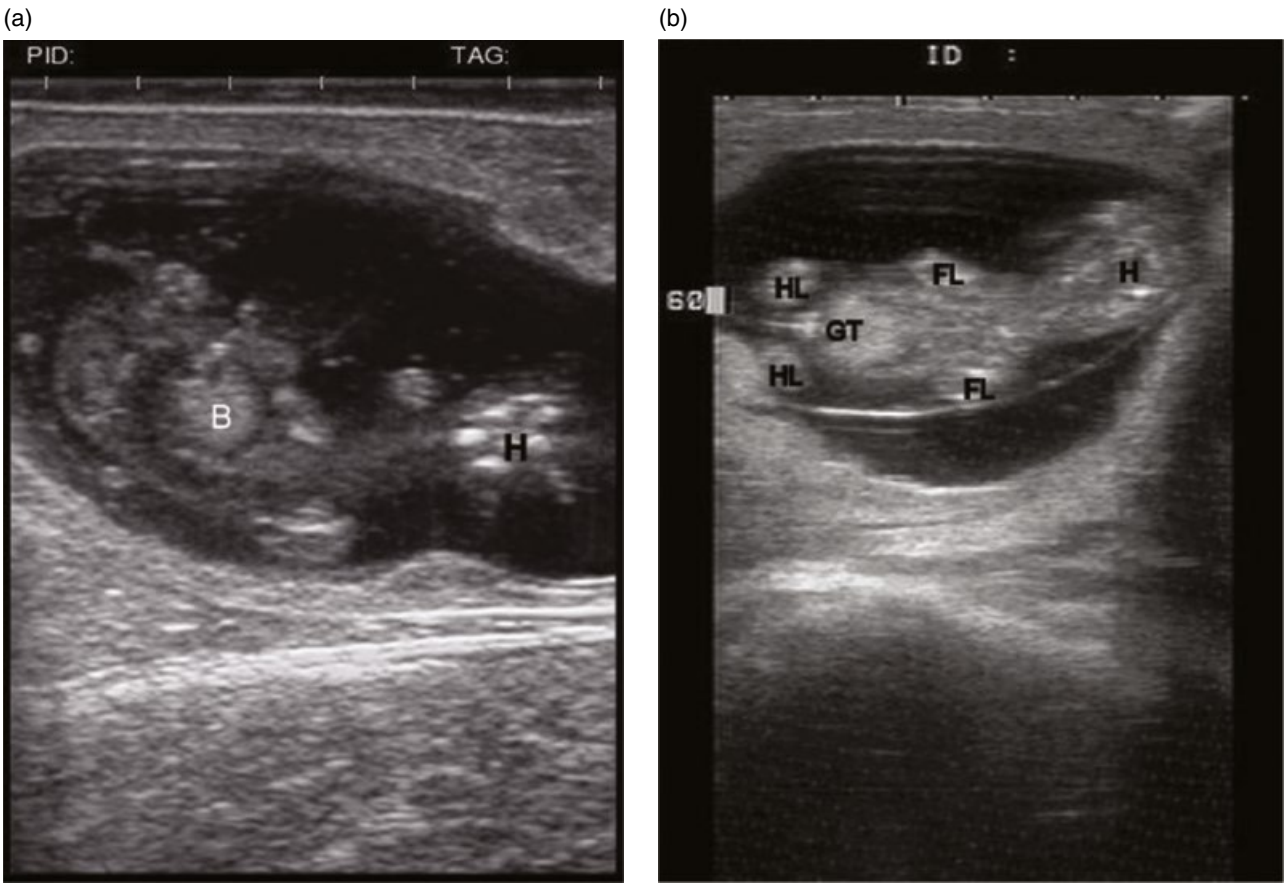


Figure 40.3 (a) Dead 65-day fetus: head (H), body (B). (b) A 60-day male fetus: forelimbs (FL), hindlimbs (HL), genital tubercle (GT).



Figure 40.4 Fetal mummy.



Figure 40.5 Three corpora lutea (cl) on one ovary.

Despite these risks it is generally not recommended to abort twin pregnancies. The possibility of failing to produce another pregnancy or of producing another set of twins makes it preferable to monitor and manage the cow carrying twins. This can include additional examinations to identify pregnancy loss, drying the cow off early to compensate for probable early calving, feeding a higher energy ration to improve body condition, providing extra monitoring and assistance at calving, and being alert for periparturient metabolic disease. In order to provide these management techniques it is important to the producer to know which animals are carrying twins. Manual palpation is not an accurate method for diagnosis of twins [24]. However, a careful ultrasonographic examination can identify most cases.

Because over 95% of twins in cattle are dizygous [25], it is helpful to examine the ovaries for two or, occasionally, more corpora lutea. Approximately 60% of pregnant cows with two corpora lutea will have twins [26]. When twins are suspected a thorough examination of the entire uterus should be performed. It is imperative to follow the tract in a systematic fashion to avoid inadvertently counting a single fetus twice. Triplets are rare but should be considered when three or more corpora lutea are present (Figure 40.5).

During examination of the uterus a twin line (TL) may be seen (Figure 40.6). This line represents the confluence of the chorioallantoic membranes of each fetus. It can be distinguished from the AV because it moves away from the fetus rather than encircling it. It should also not be confused with the umbilicus, which is not readily visible prior to 45 days and which is much thicker after that. A TL may be seen with either ipsilateral or contralateral twins. In some cases it is not seen due to the angle of examination. In later pregnancy the TL is difficult to identify because all the placental membranes are elongated and folded within the uterus. Twin pregnancies also tend to have more fluid than single pregnancies and often extend beyond the pelvic brim sooner. However, the only cardinal sign of twin pregnancy is the presence of two fetuses.

Twin pregnancies, particularly if ipsilateral, have higher rates of loss (Figure 40.2b) throughout gestation, but especially in the first 60 days before placentation is complete. This loss may be 3.1–6.9 times higher than that for singleton pregnancies [15, 27]. However, one unpublished study (A. Scheidegger, personal communication, 2005) indicates that loss of contralateral twins may be more likely in mid gestation. In either case, recheck examinations are warranted to identify cases of loss.

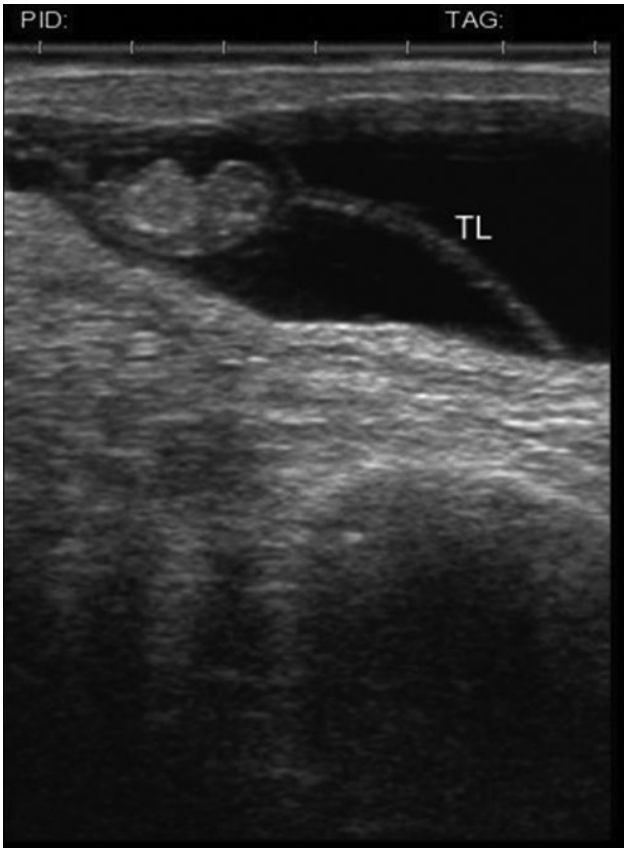


Figure 40.6 Twin line (TL).

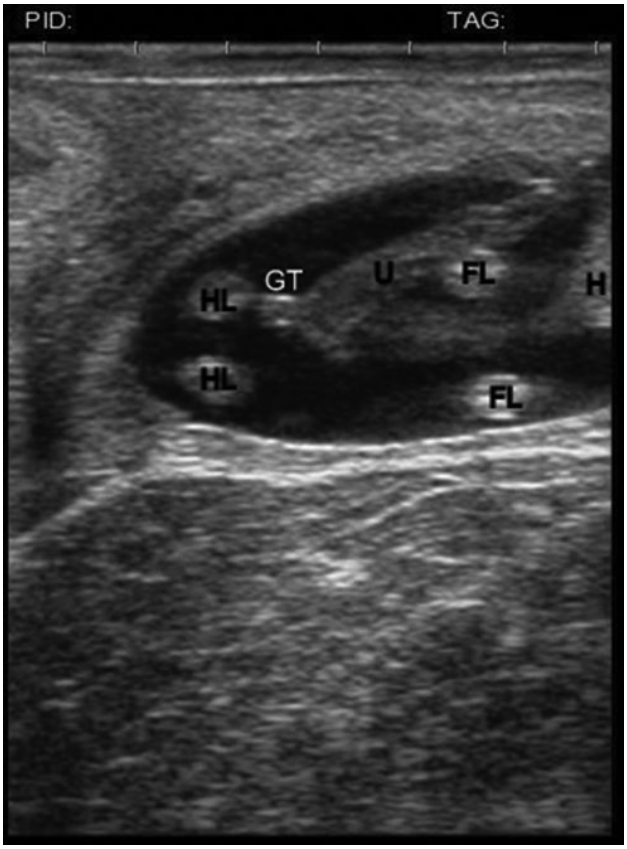


Figure 40.7 A 73-day male fetus: head (H), forelimbs (FL), hindlimbs (HL), genital tubercle (GT), umbilicus (U).

Spontaneous reduction of twin embryos has been documented [16, 21], most commonly in the late embryonic or early fetal stages. Depending on the study, 8–24% of twins reduced to a single fetus. The varying incidence is probably due at least partly to different days after insemination for initial and follow-up examinations. CL reduction often occurs in the ovary ipsilateral to the uterine horn suffering embryo reduction. Attempts to manually reduce twins in cattle have been, at best, only marginally successful [28]. In the future these results may be improved upon by ultrasound-guided needle aspiration of earlier embryos, as is done successfully in mares.

Fetal Gender Determination

Ultrasonographic gender determination of the bovine fetus was first described by Curran et al. [29] in 1989. It was found that the relative location of the genital tubercle (GT), which will become the penis in the male and the clitoris in the female, could be used to determine fetal gender beginning at day 55 of gestation. By this stage the GT has reached its final location immediately behind the umbilicus in the male (Figures 40.3b and 40.7) or immediately under the tail in the female (Figure 40.8). Until about 80 days of gestation the GT usually appears as a highly echogenic bilobed structure in both genders, but may appear mono- or trilobed.

After 70 days of gestation the skin of the prepuce or vulva begins to cover the GT [2]. At this point the adult term for the male or female external genitalia replaces the term "genital tubercle." By about 80 days the skin of the prepuce or vulva has developed enough to reduce the bright bilobed appearance of the genital structure, as shown in Figure 40.9. Also by this time the scrotum and mammary glands can be detected with most field ultrasound units. The scrotum may appear as three bright lines representing the lateral walls and median raphe or it may appear as a soft tissue sac. Teats appear as bright dots and may resemble the scrotum when it presents as three bright lines. High-quality ultrasound units can detect teats on bull fetuses, especially after about 90 days. Figure 40.10 shows teats on the same 98-day bull fetus shown in Figure 40.9. For these reasons it is not advisable to diagnose fetal gender based on presence of a scrotum or teats without visualizing the GT, penis, or vulvar structures.

After 90 days the uterus may be over the brim of the pelvis and difficult to reach for gender determination in some animals. In others it may be possible to reach the fetus at 120 days or even later; 60–80 days is the ideal time



Figure 40.8 A 65-day female fetus: tail (T), genital tubercle (GT), hooves (H).

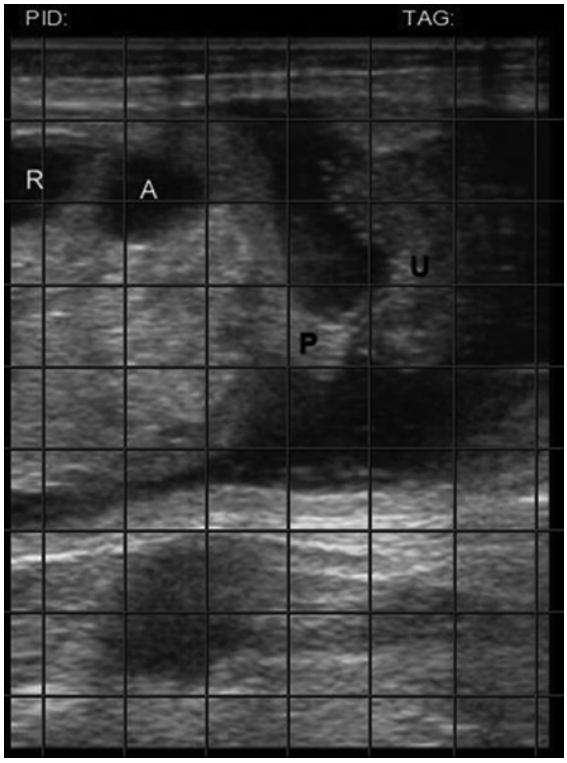


Figure 40.9 A 98-day male fetus demonstrating penis covered by prepuce: rumen (R), abomasum (A), penis (P), umbilicus (U).

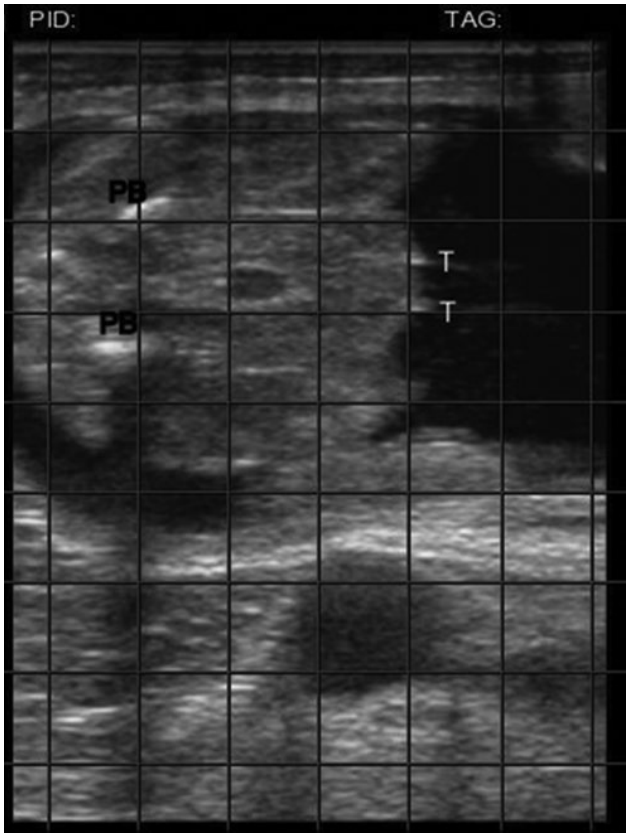


Figure 40.10 A 98-day male fetus demonstrating teats: teats (T), pelvic bones (PB).

for gender determination. At this stage the fetus is easily reached, small enough to orient the location of structures, and the GT is not obscured by skin.

Knowledge of fetal anatomic landmarks helps to locate the GT. The large tortuous umbilicus is easily identified (Figure 40.7) and the area immediately behind it can be examined for presence of a male GT. The fluid-filled anechoic rumen and abomasum (Figure 40.9) are also in the region of the male GT. The "V" shape of the ribcage permits easy identification of cranial and caudal aspects of the fetus. The head is easily identified by its echogenic bony structure.

The most common error in fetal gender diagnosis occurs because the operator does not appreciate how much the genital structures protrude from the body wall. For example, in a precise cross-sectional examination of a female fetus only the monolobed tailhead and female GT will be seen because they protrude away from other pelvic and hindlimb structures. Similarly, in the case of a male in precise longitudinal plane, only the male genital structure and the umbilicus will be seen. Therefore it is important not to stop short when scanning the fetus for gender determination.

It is also easy to move too quickly when scanning for fetal gender. There is no gap between the umbilicus and male GT

by 60 days of gestation. Likewise, there is very little space between the female GT and tailhead by that stage. Very slow, small movements are critical to avoid passing through these structures before the eye can appreciate them.

Other errors occur when another structure or artifact is mistaken for the GT. Legs sometimes occlude the umbilical area and the bones in cross-section can resemble the GT. Specular reflections in the umbilical cord can be mistaken for a male GT. Figure 40.11 shows the female GT to the right of the image, along with two less-distinct bright lines in the abdominal region where the male GT would be found. These lines are a specular reflection artifact between the fluid vessels and the walls of the umbilicus (see section "Artifacts" for further explanation). A cross-section of the tail can be mistaken for a female GT. When in doubt it is wise to examine both the umbilical region and the perineal region to confirm gender.

Finally, one must develop a quick eye to accurately identify landmarks and the GT. Because the cow and the fetus are moving it is usually not possible to isolate the structures for prolonged viewing. Training videos such as Brad Stroud's *Bovine Fetal Sexing Unedited* are valuable for training the eye to detect fetal gender quickly and accurately [30].

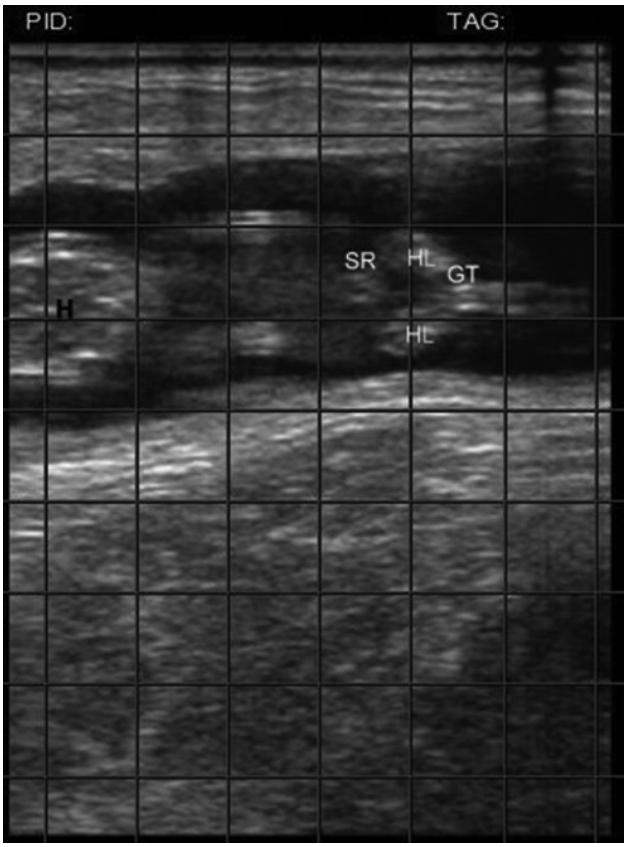


Figure 40.11 A 60-day female fetus with specular reflection artifact: head (H), specular reflection artifact (SR), hindlimbs (HL), genital tubercle (GT).

Normal Uterine and Ovarian Structures Throughout the Estrous Cycle

Proestrus

In proestrus the uterus becomes more heterogeneous as blood flow increases [31]. Anechoic mucus begins to accumulate in the lumen about three days before ovulation. One, or sometimes more, large (16–20 mm) follicles will be present. The CL is usually still visible but is becoming smaller and more heterogeneous.

Estrus

Figure 40.12 shows a normal estrus uterus in cross-section. The uterus is most heterogeneous at this point. Varying amounts of anechoic mucus are present in the uterine lumen. The large difference in acoustic impedance between the dense endometrium and the fluid mucus often creates specular reflection artifacts on the inner wall of the endometrium. The endometrium thickens to about 2.5 times its normal volume under the

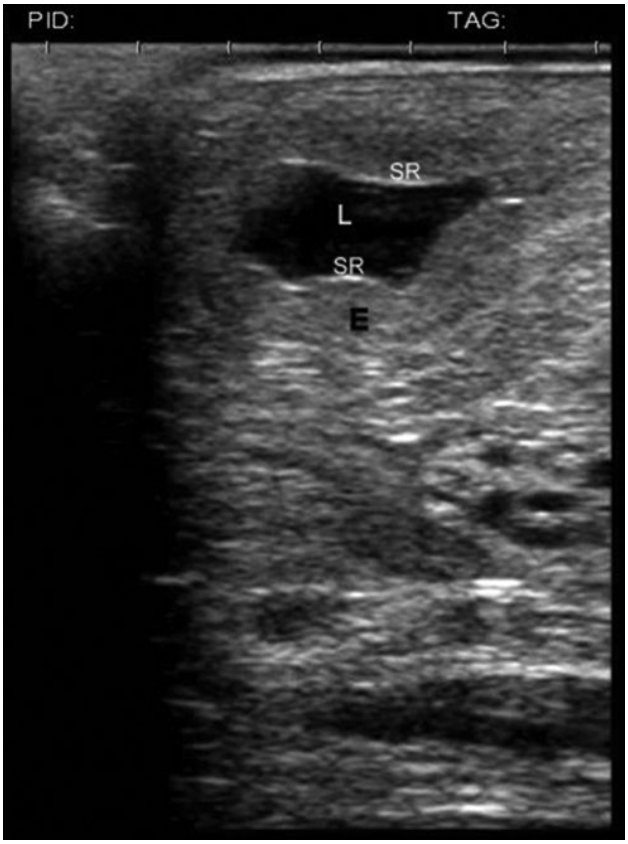


Figure 40.12 Estrous uterus in cross-section: lumen (L), endometrium (E), specular reflection artifacts (SR).

influence of estrogen. When measured in cross-section at about 2 cm from the internal uterine body bifurcation, the endometrial thickness has been shown to increase to about 9.4 mm in estrus from 6.9 mm in diestrus [32]. A mature follicle is still present until ovulation at approximately 12 hours after the end of standing heat. Sometimes a portion of the follicular fluid is not expelled during ovulation. In these cases it is difficult to determine if ovulation has occurred. A small heterogeneous CL may still be seen throughout proestrus and estrus.

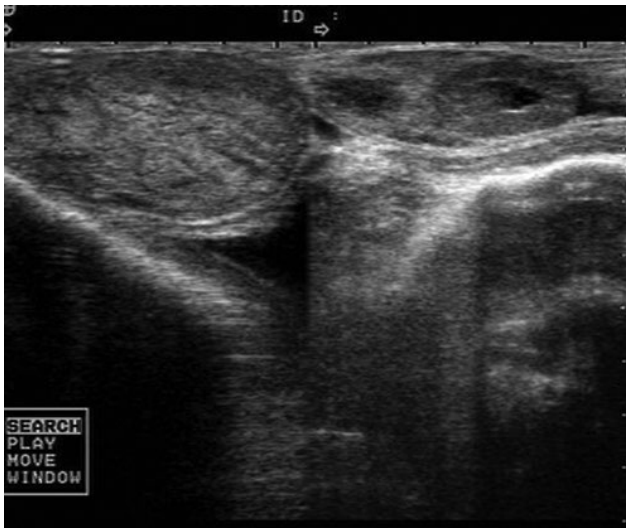


Figure 40.13 Split screen image of diestrous uterus in longitudinal section on the left and ovary with CL on the right.

Metestrus

After ovulation a new wave of small follicles (<8 mm) develops. In the first day or two of the cycle these small follicles are all that is visible on the ovaries. By day 2 or 3 a small heterogeneous CL becomes visible, often with an anechoic fluid-filled cavity. Occasionally the cavity is filled with very hyperechoic material, probably clotted blood.

Diestrus

Figure 40.13 shows a normal diestrus uterus and ovary. During diestrus the uterus is homogeneous and a medium shade of gray. There should be little or no fluid in the lumen. The CL will also be homogeneous and a medium shade of gray. The CL may or may not have a fluid cavity. Figure 40.14 shows three corpora lutea in heifers seven days after standing estrus. Despite their different appearances these are all normal corpora lutea for that stage. About 80% of corpora lutea have a cavity at some stage in development, usually early [33]. The cavity may be filled with clear fluid (Figure 40.14b), probably due to incomplete expulsion of follicular fluid during ovulation. Dense material in the cavity (Figure 40.14c) may be clotted blood remaining after ovulation. The fluid cavity tends to fill in over time, but may persist into pregnancy. A mature CL with a fluid cavity may also have bright specular reflection artifacts where the dense, fully developed luteal tissue meets the clear fluid cavity (Figure 40.15). These artifacts are less evident in less

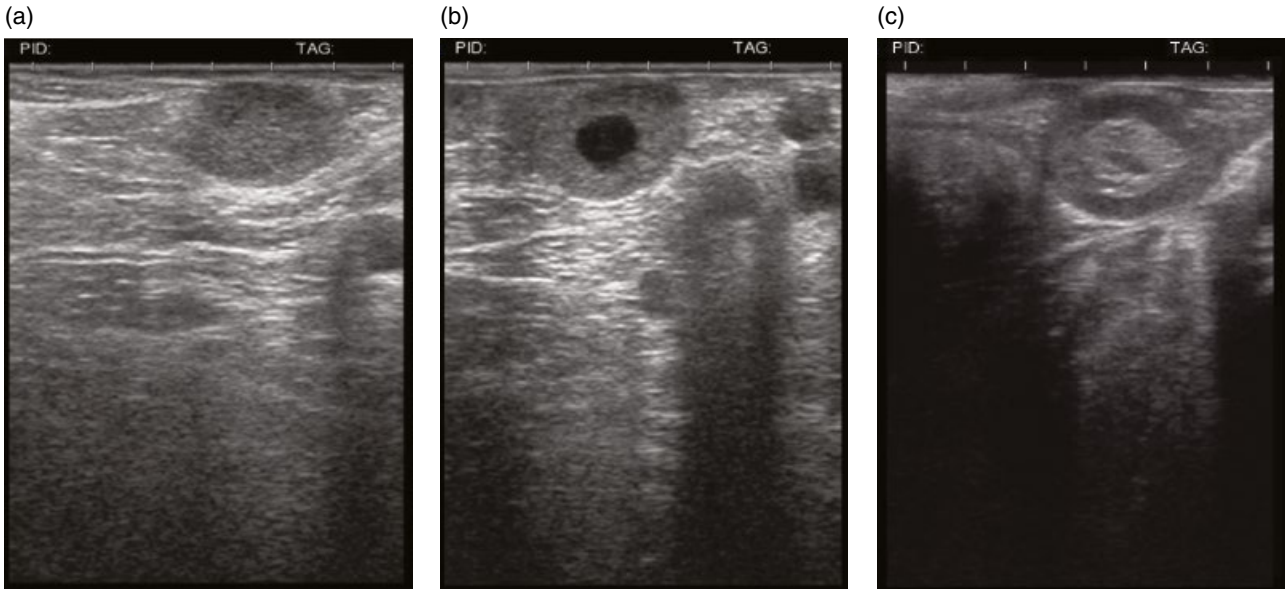


Figure 40.14 (a) Solid CL seven days after estrus. (b) Cavitary CL seven days after estrus. (c) Cavitary CL with dense center seven days after estrus.



Figure 40.15 Mature cavitary CL with specular reflections.

mature cavitary corpora lutea (Figure 40.14b) because there is not yet adequate acoustic mismatch for them to occur. Specular reflection artifacts are discussed further in the section on "Artifacts." Follicles ranging in size from 2 to 20 mm may be present depending on the stage of follicular wave cycles.

Pregnancy

Before 21 days of gestation the uterus and ovaries look much as they do in diestrus. A small amount of fluid may be visible in the uterine lumen at 21–23 days of gestation. The embryo is difficult to see under field conditions until at least 24 days. The gravid uterus does not have a thickened endometrium as in estrus. Specular reflections are either not present or much less pronounced than in estrus. The CL is usually solid at this point, but may be cavitary.

Ovarian Pathology

Anovulation

Wiltbank et al. [34] have classified ovulation failure into four basic categories. Ultrasound can assist in determining the category of ovulation failure.

Anovulation with Follicle Growth Only to Emergence

The term "static ovaries" has been used to describe conditions in which follicles grow to emergence (about 4 mm) but do not mature further to ovulatory size of 8 mm or more. On ultrasound examination this condition resembles early metestrus, with many small follicles, no follicles over 4 mm, and no visible CL. A second examination a few days later is required to differentiate this condition from metestrus. According to Wiltbank, this type of anovular condition is rare except in extreme malnutrition and possibly in Zebu cattle.

Anovulation with Follicle Growth to Selection but not Ovulatory Size

This type of follicular growth pattern is typical in the postpartum period. There is no CL and the ovaries are small, but frequent ultrasound examinations reveal that follicles grow up to 8.5 mm in a dynamic wave pattern.

Anovulation with Follicle Growth to Ovulatory or Larger Size (Follicular Cysts)

Follicular cysts have been defined as thin-walled fluid-filled follicular structures greater than 22 mm in diameter [35]. A follicular cyst (Figure 40.16) is differentiated from a cavitary CL, luteal cyst, or luteinizing follicular cyst (Figure 40.17) by the absence of a visible luteal rim. Follicular cysts are benign in most cases, with luteal structures and follicular waves developing normally on the same or contralateral ovary. Benign cysts can persist throughout several cycles and into pregnancy. Ultrasound is far superior to manual palpation for diagnosis of benign versus pathologic follicular cysts because it is more capable of identifying luteal tissue within the fluid-filled structure or the presence of a small CL elsewhere [36, 37].

Thorough ultrasonographic examination of both ovaries is essential to properly classify follicular cysts. If luteal tissue is detected the cyst is benign. Figure 40.18 shows a large ovary with several follicular cysts and a normal CL at 5 o'clock. If no luteal tissue is seen, a second examination must be performed in about 5–14 days to determine if a CL has developed. If not, the cyst is likely preventing normal cyclicity.

Follicular cysts have traditionally been treated with GnRH. Following treatment, most follicular cysts luteinize rather than ovulate. In these cases, examination seven days later will reveal that the original thin-walled structure has developed a thicker rim of luteal tissue (Figure 40.17). This large soft structure will still feel like a follicular cyst on manual palpation, but the luteal tissue is clearly seen with ultrasound. Alternatively, the cyst may remain but another, smaller follicle may be induced to ovulate. In this case the original follicular cyst will still be seen after seven days, but a

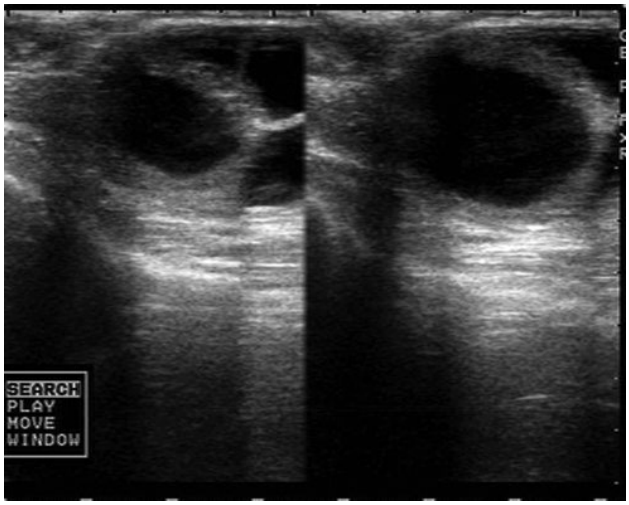


Figure 40.16 Multiple follicular cysts.



Figure 40.17 Luteinizing follicular cyst.

small CL will be evident elsewhere. It has been demonstrated that the presence of progesterone improves conception rates to the Ovsynch synchronization program [38]. Hence animals that respond to GnRH treatment by producing luteal tissue may be started on this protocol with good success.

Another common treatment for follicular cysts is progesterone supplementation in conjunction with the Ovsynch synchronization protocol at the time of diagnosis.



Figure 40.18 Benign follicular cysts with CL.

Progesterone supplementation adds expense and inconvenience. In some countries it is not permitted or requires substantial documentation. For these reasons, ultrasound diagnosis is very helpful to limit progesterone supplementation only to cystic cows that do not produce luteal tissue in response to GnRH treatment.

Anovulation in the Presence of Luteal Tissue

Prolonged luteal function prevents ovulation at normal intervals. Various papers define prolonged luteal function differently. Proposed definitions include, but are not limited to, the persistence of the CL with milk progesterone above 3 ng/ml for more than 30 days [39], or milk progesterone above 3 ng/ml for at least 19 days [40]. Frequent multiple ultrasound examinations or progesterone tests are required to confirm persistent luteal function. Because the Ovsynch protocol works well in the presence of progesterone, it is more important to identify the presence of a CL rather than attempting to identify if it is persistent.

Other Ovarian Pathologies

Ovarian pathologies other than the above anovular defects are rare in cattle. Granulosa cell tumors are sometimes

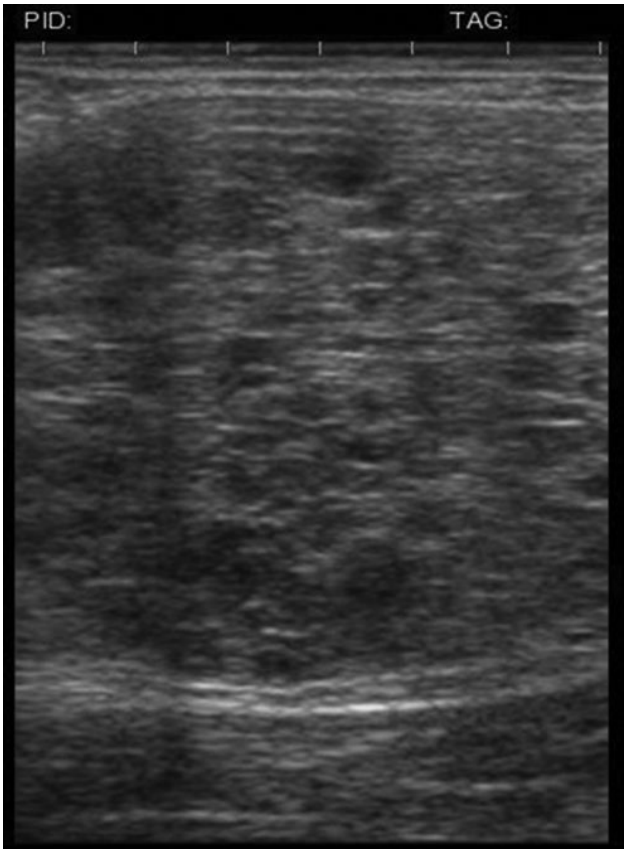


Figure 40.19 Granulosa cell tumor.

seen (Figure 40.19). The ovary is very large. The hyperechoic areas are specular reflections between the vasculature of the tumor and the denser surrounding tissue.

Synchronization Protocols

When cattle are presynchronized with prostaglandin (Presynch) before beginning Ovsynch, all normal cycling animals should have a CL when the first GnRH injection (G1) is scheduled. An examination of ovaries at this time will reveal if presynchronization was successful. If many cows do not have corpora lutea at this time, the protocol has failed and the reason must be found. Poor compliance, poor cow health, low body condition scores, and nutrition problems are all possible reasons for failure.

It has been shown that the presence of a CL and the availability of an ovulatory size (≥8 mm) follicle at G1 improve conception rates for Presynch/Ovsynch [38]. This study is summarized below:

- For cows with a CL and ovulation to G1, the conception rate was 42.2% when pregnancy examination was performed at 38 days.
- For cows with a CL and no ovulation to G1, the conception rate was 37.7%.
- For cows with no CL and ovulation to G1, the conception rate was 27.6%.
- For cows with no CL and no ovulation to G1, the conception rate was 15.4%.

Ultrasound is very sensitive for identifying the presence of a CL, including very early metestrus corpora lutea and regressing proestrus corpora lutea. These very early and late corpora lutea are probably not producing progesterone in adequate levels for optimal conception rates to Ovsynch. Therefore it has been suggested that a cutoff size be used to determine if a CL is functional. Bicalho et al. [37] found that ultrasound was nearly 90% sensitive and specific for identifying a CL producing more than 1 ng/ml blood progesterone if a 2.2-cm cutoff size was used. Palpation, by contrast, was 33–60% sensitive and 77–93% specific for identifying blood progesterone greater than 1 ng/ml, varying among palpators.

Ovsynch works best from days 5 to 10 of the cycle when some early corpora lutea may not yet be 2.2 cm in diameter. Therefore this author uses a minimum cutoff size of 1.5 cm if the CL appears to be a typical early diestrus CL (see section on "Normal Ovarian Structures Throughout the Estrous Cycle"). If the CL appears to be regressing during proestrus, it is more desirable to wait until 5–10 days after ovulation to begin the Ovsynch protocol.

Based on the findings in the study by Galvão et al. [38], the presence of a functional CL was more important than ovulation to G1. However, some ultrasonographers [41] feel it is also of value to identify the presence of a potentially ovulatory follicle greater than 8 mm. Given that it is difficult to assess if follicles of this size are normal, persistent, or atretic, this author relies more on the presence of a CL of appropriate size and stage.

Uterine, Cervical and Vaginal Pathology

Metritis, Endometritis, and Pyometra

Clinical and most subclinical uterine infections can be diagnosed with ultrasound. Field ultrasound units can visualize 2 mm of fluid, far less than detected by manual palpation. Ultrasound can also evaluate the character of the fluid based on echogenicity. In most cases of metritis/endometritis, echogenic purulent material can be seen in the uterine lumen. This fluid may be any shade from light gray to very bright white depending on its density. Intraluminal fluid in cases of pyometra is usually voluminous and of medium echogenicity (Figure 40.20).



Figure 40.20 Pyometra.

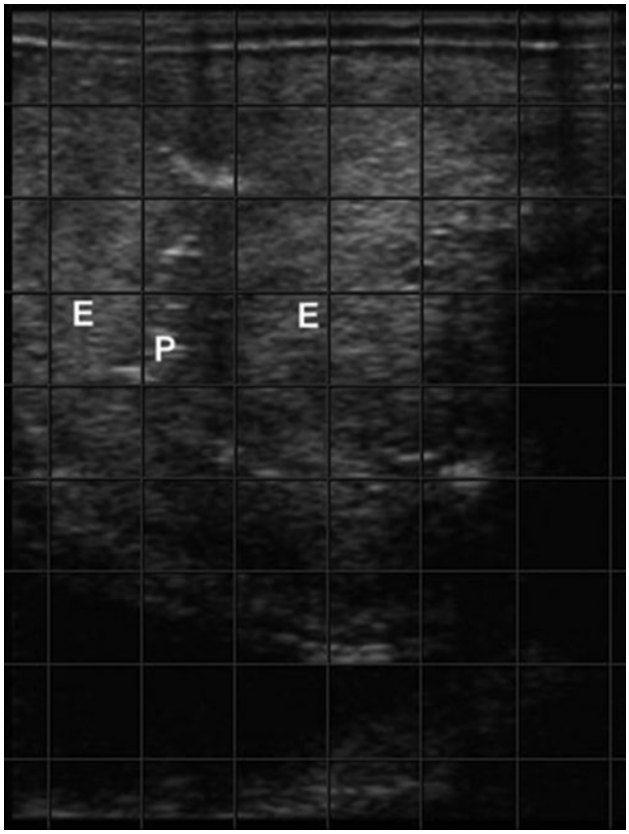


Figure 40.21 Subclinical metritis: endometrium (E), purulent material (P) in lumen.

Cases of chronic subclinical metritis/endometritis usually have little intraluminal fluid of higher echogenicity (Figure 40.21). The ultrasonographer should be careful not to confuse small volumes of echogenic intraluminal fluid with specular reflections due to estrus (Figure 40.12). When fluid is not detected, increased endometrial and cervical thickness may be used to evaluate endometritis in the early postpartum period [42]. The ultrasonographer should keep in mind that endometrial thickness is also increased in estrus and must perform a thorough examination of both ovaries and uterus to make a differential diagnosis.

Mucometra

Mucometra is an accumulation of sterile anechoic or slightly echoic fluid in the uterine lumen not associated with pregnancy. Specular reflections may or may not be present. The endometrium is not thickened as it is in estrus. Mucometra does not appear to be related to ovarian structures. The ovaries may have follicular cysts, normal corpora lutea, or no significant ovarian structures. It is critical to confirm the absence of an embryo or fetus in cases of mucometra. When in doubt the ultrasonographer should perform a second examination a few days later to confirm the diagnosis. Mucometra in heifers may be related to the presence of a complete hymen or other congenital blockage of the tubal portions of the reproductive tract. The cause in adult cows is unknown.

Lymphosarcoma

Uterine lymphosarcoma is identified by very firm thickening of the uterine wall that may be localized or throughout the entire uterus. The affected uterine tissue (Figure 40.22a) looks like the tissue of a lymph node (Figure 40.22b).

Abscesses

Uterine, cervical, or vaginal abscesses can be caused by difficult parturition, rough assistance during parturition, or trauma from artificial insemination. They can be distinguished from metritis, cervicitis, or vaginitis by the presence of a capsule around the purulent material (Figure 40.23). Uterine abscesses have a poor prognosis for future fertility. Cervical abscesses may or may not be of concern. Careful ultrasound examination can determine if the abscess extends into the cervical lumen or is entirely outside it. If the latter, the

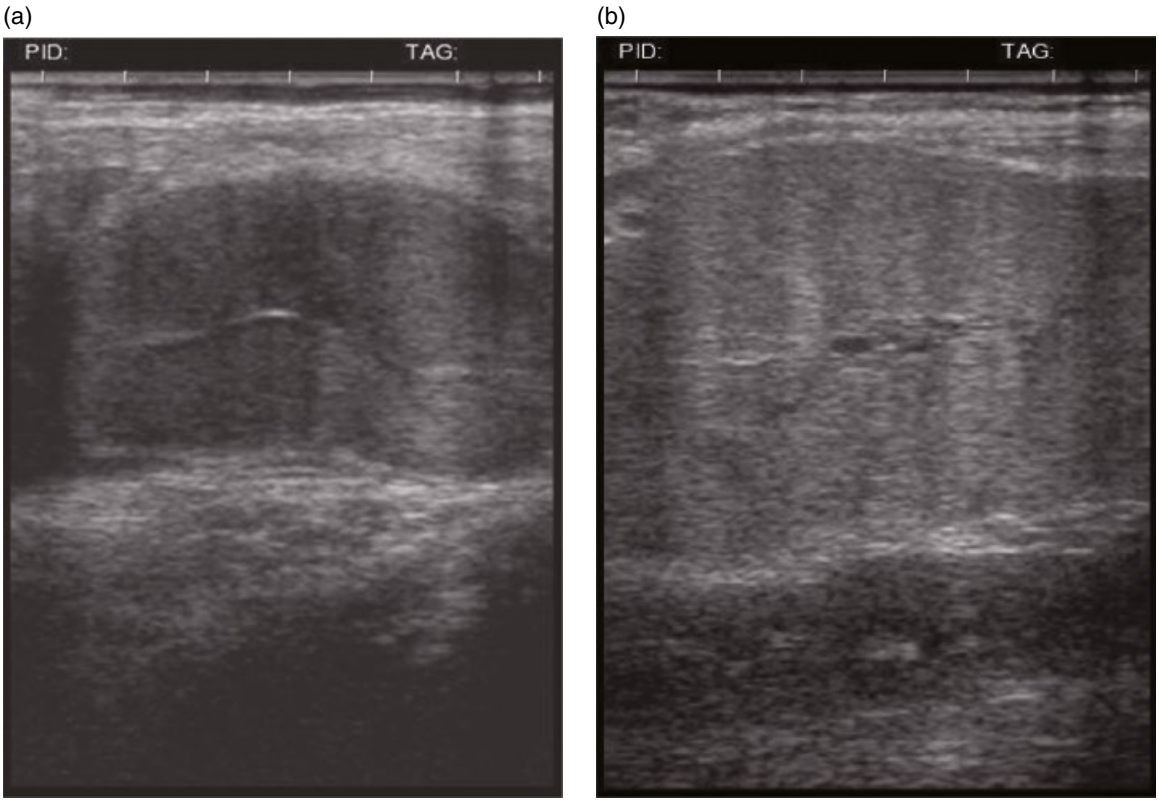


Figure 40.22 (a) Uterine lymphoma. (b) Pelvic lymph node.



Figure 40.23 Uterine abscess: capsule (C), purulent material (P).

prognosis for future fertility and parturition is good. Vaginal abscesses generally do not cause difficulty unless they are large enough to interfere with breeding or parturition.

Vaginitis

The normal vagina presents as a thin white line representing the confluence of opposite sides of the vaginal wall. In cases of vaginitis echogenic material is visible in the vaginal vault and the vaginal mucosa is thickened. Unless echogenic material is also present in the uterus, the prognosis for fertility is fair to good if a protective sheath is used on the insemination gun at breeding. Vaginitis may be primary or caused by urine pooling (UP) (Figure 40.24).

Vaginal Urine Pooling

Urine is usually less echogenic than purulent material, but is often mixed with purulent material when accumulated in the vagina (Figure 40.24). As with vaginitis, prognosis for fertility is fair to good if there is no abnormal fluid in the uterine lumen and a protective sheath is used on the insemination gun at breeding. UP may be accompanied by cystitis.

Pneumovagina

Gas in the vagina or elsewhere presents as a reverberation artifact (Figure 40.25) (see section "Artifacts").

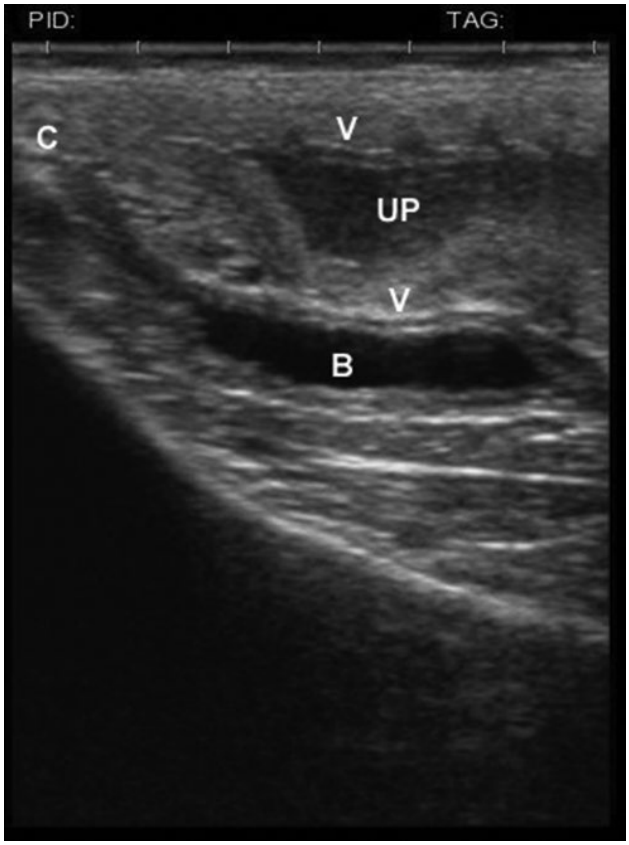


Figure 40.24 Vaginitis due to urine pooling: vaginal walls (V), urine pooling (UP), bladder (B).

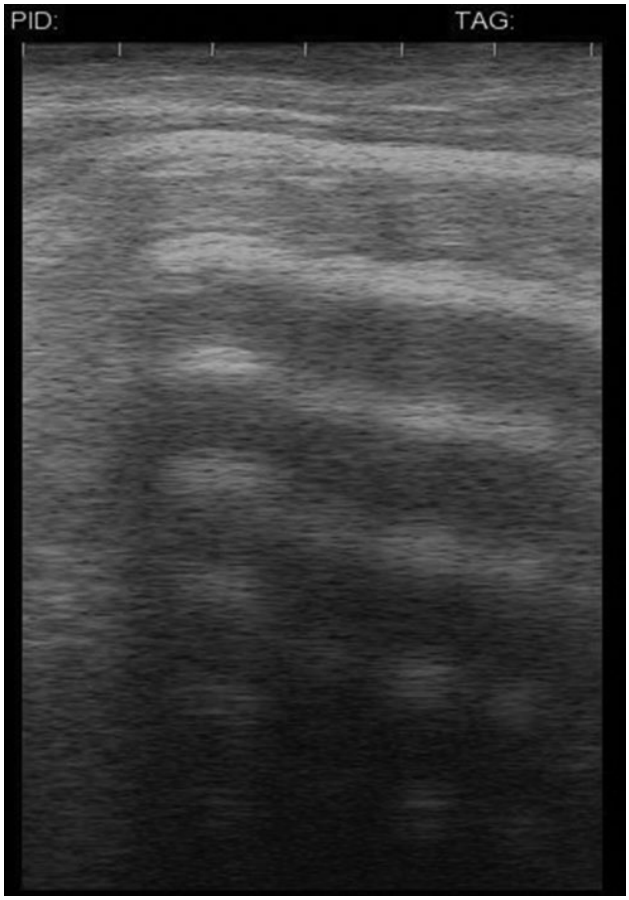


Figure 40.25 Reverberation artifact due to pneumovagina.

Embryo Transfer

Donor Evaluation

Donors should be assessed for uterine health and ovarian function before beginning the superovulation protocol. If the cow is examined during follicular recruitment, it may be possible to predict the response to future superovulation. Cows that recruit large numbers of follicles at the beginning of each follicular wave can be expected to produce more follicles after treatment with follicle-stimulating hormone (FSH) [43, 44].

It is usually not necessary to examine the donor cow on the day of insemination unless very rare or expensive semen is to be used. In those cases it is helpful to know if enough ovulatory size (≥8 mm) follicles are present to warrant insemination. Care must be taken to scan gently over the ovaries without manipulation or pressure. It is possible to rupture small follicles or even remove the ovary from the ovarian bursa with rough handling.

On collection day, ultrasound is much more useful than manual palpation for counting the number of corpora lutea [38]. Small corpora lutea, cavitary corpora lutea, and corpora lutea imbedded deep in the ovary can be seen. Figure 40.26 shows two sections of the same ovary. Note the solid corpora lutea in the first section and the cavitary corpora lutea or unovulated follicles in the second section. The ultrasonographer should keep in mind that some cavitary corpora lutea will have very thin walls and may be difficult to differentiate from unovulated follicles [45]. When these fluid-filled structures are seen the cow should still be flushed. It is very possible that more embryos than expected will be retrieved.

Recipient Evaluation

Recipients should be evaluated for uterine health and ovarian function prior to beginning estrous synchronization. This is more important for adult cows than for heifers due to increased risk of uterine or ovarian pathology in parous animals.

It is critical that ultrasound is used to identify corpora lutea before embryo transfer (Figure 40.14). Because ultrasound is much more accurate than manual palpation for identifying small or fluid-filled corpora lutea [37, 45], more animals will be used. Almost 100% of corpora lutea in recipients are diagnosed with ultrasound, but only 80% with manual palpation. Maintaining open animals is costly, so it is imperative to identify all appropriate recipients. Examination for uterine health should

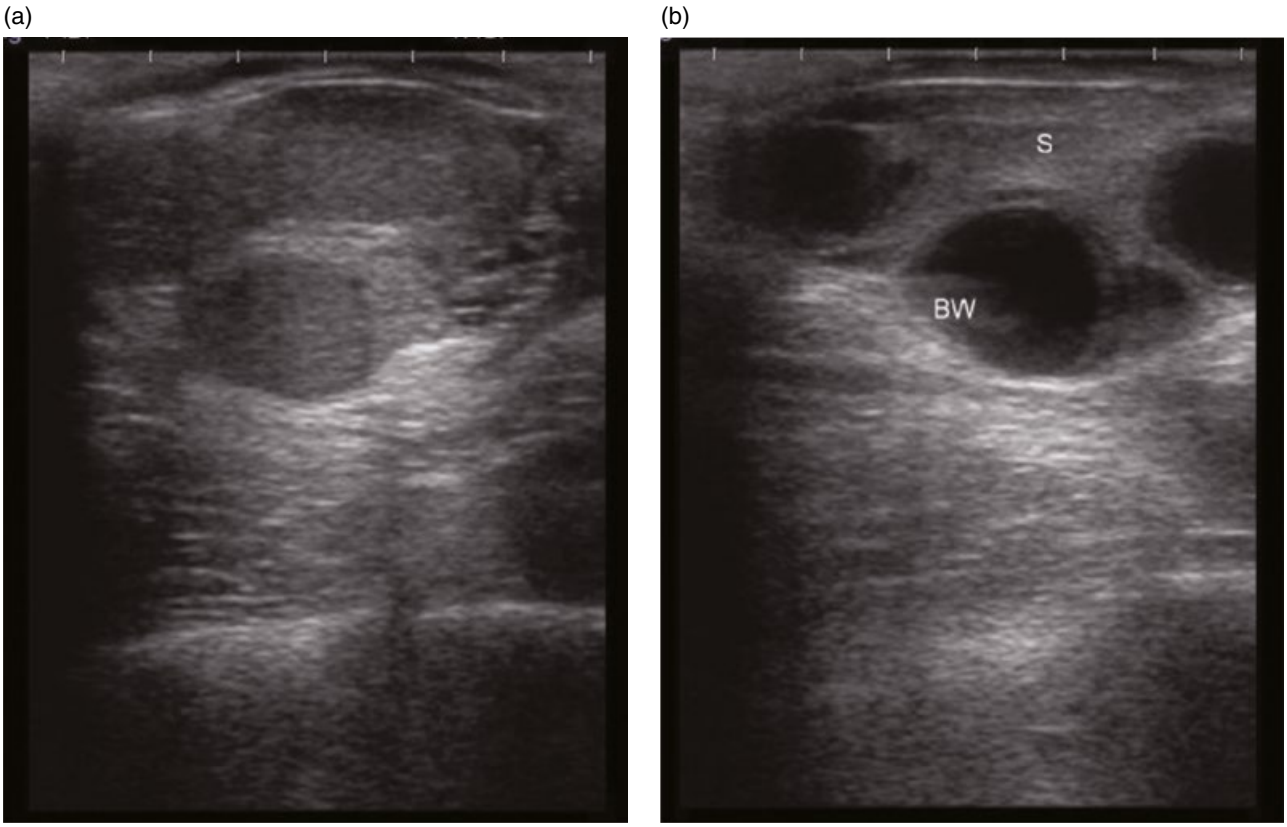


Figure 40.26 (a) Multiple corpora lutea on embryo flush day. (b) Cavitary corpora lutea or unovulated follicles on embryo flush day: ovarian stroma (S), beam width artifact (BW).

also be performed at this time, especially if not evaluated prior to synchronization.

Pregnancy examination of recipients can take place at any stage comfortable for the ultrasonographer. I have noted that some frozen embryo pregnancies appear one day smaller than fresh embryo pregnancies. Also, some *in vitro;* fertilized (IVF) embryos appear one day larger. This is of no concern as long as the embryo is viable and large enough to be detected on the day post-transfer chosen for examination. Twins are rare in embryo transfer pregnancies, but are at high risk for loss when they occur. Twins may be due to splitting of the embryo, intentional transfer of two embryos, or insemination of the recipient during estrus prior to embryo transfer.

An ideal time to perform pregnancy examination is at 21 days after transfer (28 days in embryonic development). At this stage open animals with no pathology can immediately receive an embryo if the CL is appropriate. In dairy animals, this is probably more effective in heifers than adult cows. Adult dairy cows often do not have typical 21-day estrous cycles [46], so may not be at the correct stage of luteal development.

Recheck pregnancy examinations after 60 days of gestation should always be performed due to the high value of most embryo transfer and IVF pregnancies. Fetal gender determination and evaluation for fetal anomalies can also be done at this time.

Ovum Pick-Up

Collection of ova for IVF is done with a guided needle attached to a transvaginal ultrasound transducer. A curvilinear transducer is usually used, but a standard linear rectal transducer can be adapted for the purpose if attached to a special introducer containing the guided needle.

Embryonic and Fetal Anomalies

Embryonic and fetal anomalies are rare but of great importance. Depending on the anomaly, it may be desirable to monitor the pregnancy, abort the pregnancy, or take special precautions at parturition. Most anomalies are best seen at about 50–90 days, when the fetus is developed enough to resemble a small version of an adult bovine, but not too large to reach for a thorough examination. A few abnormalities can be detected earlier.

Abembryonic Vesicle

Figure 40.27 shows a 35-day embryo that is outside the AV. Such embryos rarely survive even if they have a heartbeat on the day of first examination. Recheck examination usually reveals an empty amniotic sac or complete lack of uterine contents.

Schistosomus Reflexus

Note the fluid-filled fetal stomach (FS) outside the body cavity in Figure 40.28. In most cases the spinal column (SC) is severely bent, sometimes into a "V" shape.

Fetal Ascites

Note the fluid within the peritoneal cavity in Figure 40.29. In a normal fetus the abdominal organs should completely fill the cavity with no visible fluid. In some cases there is also fluid around the heart or lungs. Careful assessment should be made to differentiate fetal ascites from a dead fetus with fluid accumulation (see Figure 40.3a).



Figure 40.27 Abembryonic vesicle.

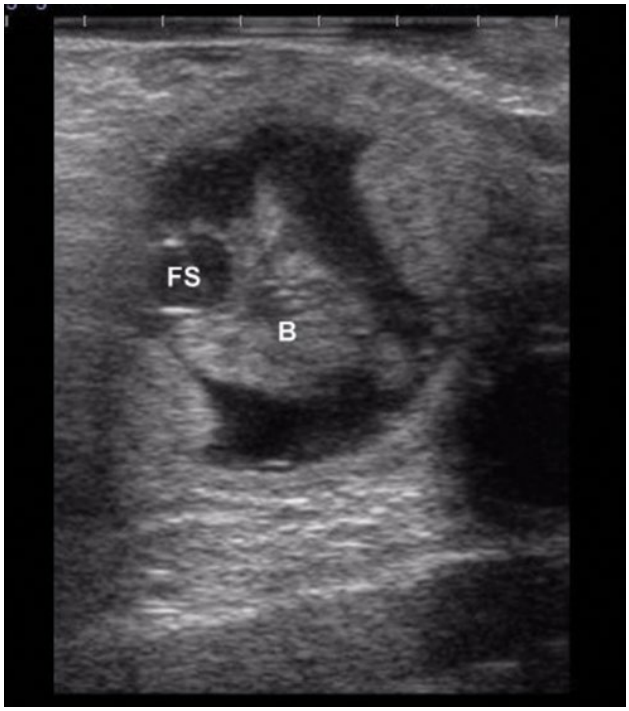


Figure 40.28 Schistosomus reflexus: fetal stomach (FS), fetal body (B).

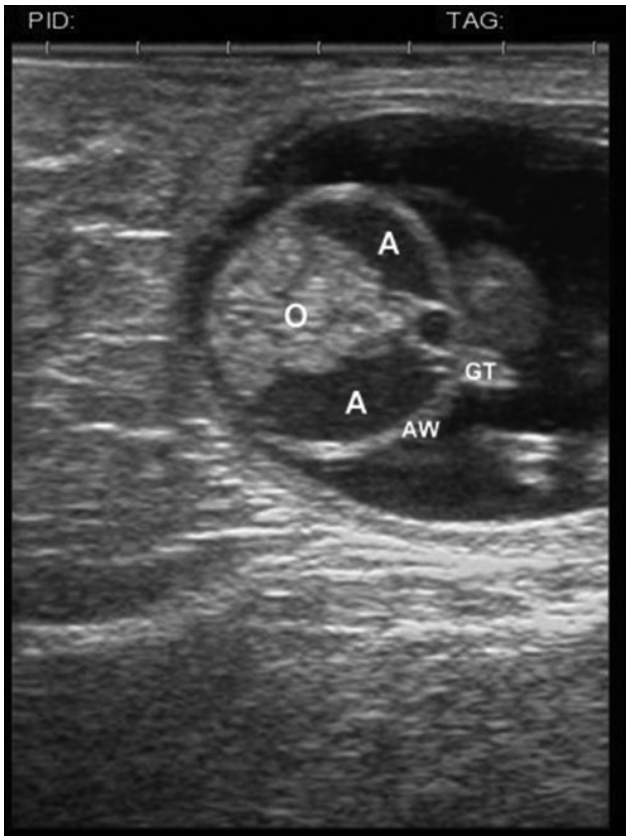


Figure 40.29 Fetal ascites: abdominal organs (O), ascites (S), abdominal wall (AW), male genital tubercle (GT).

Fetal Anasarca

Compare the fluid under the skin in fetal anasarca (Figure 40.30) with the fluid within the abdominal cavity as in fetal ascites (Figure 40.29). Follow-up of fetal anasarca sometimes reveals that the fluid is no longer visibly present. In other cases there is no change or fetal death occurs.

Umbilical Hernia

Figure 40.31 shows the fetal chest to the left of the screen and a large umbilicus to the right. The umbilicus contains tissues of various densities probably representing abdominal structures. Not shown in this view is the absence of the fluid-filled FS within the abdomen.

Amorphous Globosus or Acardiac Twin

The round structure to the right of the normal fetus in Figure 40.32 is likely a ball of fetal tissue sustained by the circulatory system of its live twin. These anomalies usually do not grow large so are rarely a problem at parturition. They have no gender so are not of concern for freemartinism.

Conjoined Twins

Figure 40.33 shows a fetus with two heads. Conjoined twins are very rare and must be differentiated from normal twins lying close together.



Figure 40.30 Fetal anasarca: skin (SK), fluid (F), head (H), spinal column (SC).



Figure 40.31 Umbilical hernia: herniated umbilicus (UM), ribs (R).

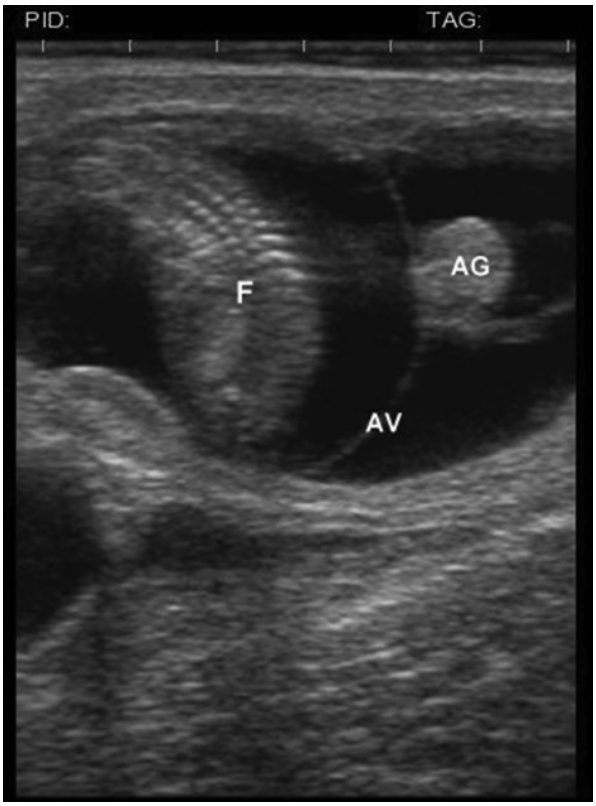


Figure 40.32 Amorphous globosus: normal fetus (F), amorphous globosus (AG), amniotic vesicle (AV).

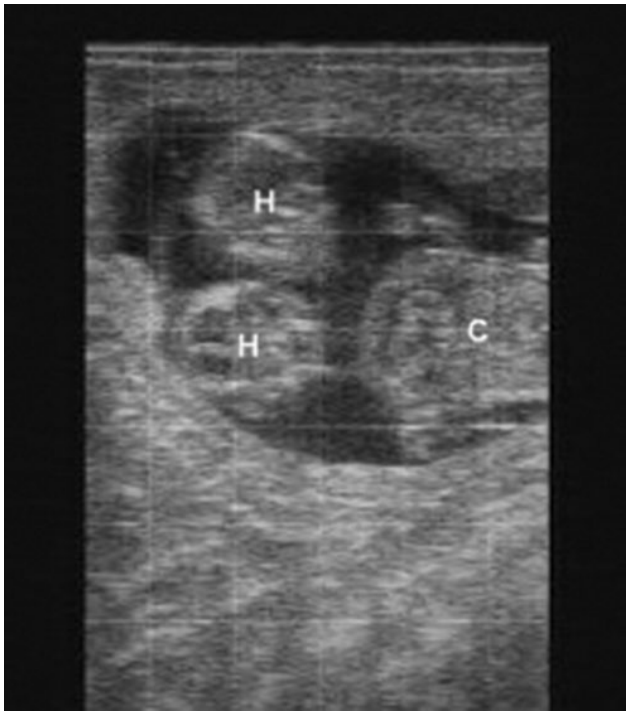


Figure 40.33 Conjoined fetus with two heads: heads (H), chest (C).

Artifacts

It is important to recognize common artifacts in order to avoid misdiagnosing them as normal structures or pathology. When identified properly artifacts are often helpful in evaluating the reproductive tract. For a more complete description of artifacts and their interpretation refer to Ginther [1].

Specular Reflection Artifacts

These common artifacts occur when two interfaces of very different densities are aligned horizontally to each other and perpendicular to the ultrasound pulse. They are most often seen when a fluid-filled structure is adjacent to denser tissue. They appear as thin bright horizontal lines of varying lengths. Figure 40.12 shows specular reflections between the turgid uterine tissue of a cow in estrus and the clear fluid in the lumen. Figure 40.15 shows specular reflections in a cavitary CL. Figure 40.19 shows many small bright specular reflections representing the confluence of the tissue and the fluid-filled vasculature of the granulosa tumor.

Reflection and Refraction Shadows

Fluid reflects and refracts ultrasound waves just as the surface of a lake reflects and refracts light waves. When the ultrasound beam is either refracted at an angle into the fluid or reflected away from the fluid, it is not echoed back to the transducer. Hence no image will be seen below the point of reflection/refraction. This artifact is commonly seen at the lateral edges of fluid-filled structures (Figure 40.34).

Beam Width Artifacts

When one ultrasound beam passes through two tissues of different densities at the same depth, a beam width artifact is produced (Figure 40.34). Two echoes reach the transducer at the same time, but only one is read. These artifacts are most common on the lateral walls of fluid-filled structures and cause the sides of the structure to appear more echogenic than the contents. Figure 40.34 shows beam width artifacts between the endometrium and fluid contents of a gravid uterus. Beam width artifacts are most obvious when using ultrasound machines with poor lateral resolution.

Beam width artifacts are also common on the edges of follicles or follicular cysts (Figure 40.26b). In these cases the artifact may be mistaken for luteal tissue. In the uterus they may appear to be abnormal contents in the lumen. In either case a slow scan throughout the structure will help differentiate the artifact from actual tissue.

Reverberation Artifacts

Repeated echoing of ultrasound waves between two strong interfaces creates reverberation artifacts. One interface is



Figure 40.34 Reflection/refraction artifacts (R), beam width artifacts (BW), enhancement artifact (E).

usually the rectal wall/transducer. The second interface is usually gas or bone. Each time the wave is echoed it is reduced by attenuation. Hence each reverberated echo is weaker than the previous one. Also, the time delay between echoes results in each later echo being seen farther down on the ultrasound screen. The resulting image on the screen consists of equidistant, increasingly smaller, parallel artifacts. Figure 40.25 shows the reverberation artifact caused by pneumovagina.

Mirror Image Artifacts

Mirror image (MI) artifacts are a type of reverberation artifact. They are most common when the pelvic bone lies below the structure being examined. Figure 40.35 shows a 60-day pregnancy with a false "twin" in MI below the pelvic bone. Understanding MI artifacts helps avoid mistaking one structure for two.

Blockage Shadows

Material dense enough to block or attenuate the ultrasound beam causes blockage shadows. Most obvious are shadows caused by bone, as in the "prison bar" artifacts shown in Figure 40.36. Less dramatic shadows can be caused by manure between the transducer and rectal wall leading to a poor-quality dark image.

Enhancement Artifacts

Enhancement artifacts are a function of machine settings. Because ultrasound beams are attenuated over time and distance, it is necessary to increase the gain (volume) at deeper levels so that similar tissues at various depths from the transducer appear the same. However, when less echogenic material such as fluid is near the face of the transducer the beam is not attenuated and structures below the fluid appear brighter than they should. Figure 40.34 shows enhancement below the fluid-filled uterus. Gain controls can be manipulated to reduce enhancement artifacts if desired by the ultrasonographer.

Crystal Defects

Crystal defects are seen as black vertical lines extending vertically from the transducer face (Figure 40.37). They can be differentiated from shadow artifacts or reflection/refraction artifacts because they always stay in the same location on the monitor and always begin at the face of the transducer.



Figure 40.35 Mirror image artifact: fetus (F), pelvic bone (PB), mirror image (MI).



Figure 40.36 Prison bar artifacts.



Figure 40.37 Crystal defects.

They may be caused by broken crystals in the transducer or by broken wires connecting the crystals to the power source in the unit. Loss of a few crystals may not be noticed, but loss of many will greatly reduce image quality.

The Future

Technologies used in human medicine are being adapted to veterinary use. Three-dimensional ultrasound has been described in mares [47] and may have advantages over two-dimensional imaging for identifying fetal anomalies and for fetal aging. Color Doppler ultrasound has been studied extensively in horses and, to a lesser extent, in cattle [48]. It is currently feasible to use color Doppler ultrasound to assess fetal viability in cattle. In the future it may be possible to use blood flow changes in ovarian structures and the uterus to more accurately assess stage of the estrous cycle and predict ovulation. Some field ultrasound units already come equipped with color Doppler capabilities in addition to standard B-mode ultrasound.

Other Uses

Ultrasound has many uses outside the scope of this chapter. Ultrasound of the male reproductive organs has been well described [49]. Other organ systems can be assessed with ultrasound for diagnosis or prognosis of disease [50].

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41

Beef Herd Health for Optimal Reproduction

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Introduction

A recent analysis of cow-calf enterprise profitability indicates that there is more variation in production costs than income among Kansas beef producers [1]. However, reproductive efficiency is still the most important output parameter affecting the financial health of these operations. While there are many reasons for suboptimal reproductive performance and calf survival, infectious disease is a major contributor and often plays a pivotal role. Reproductive disease may manifest itself in a number of ways, depending on the pathogen involved. Early embryonic death, late-term abortion, "weak calf syndrome," and delayed conception are all common clinical scenarios. However, the end result is that the operation will have fewer kilograms to market from weaned calves. It is critical that the veterinary practitioner be able to understand the relationship of these infectious agents with the risk of exposure, timing of gestational loss, herd diagnostic information, and herd productivity. Only then can comprehensive immunization programs be constructed for the entire operation that will minimize losses associated with reproductive pathogens.

Utilization of Diagnostics

It is critical that diagnostic information be utilized in the construction of the herd health program. The selection of vaccines to be used in a program depends on the presence of a particular pathogen in the herd or geographical area, its risk of introduction into the herd, and an individual producer's cattle working schedules [2]. It is important to determine the category or period of reproductive loss since reproductive pathogens have a tendency to occur within specific stages of gestation. Classically these categories have been defined as early gestational, mid gestational, late gestational, and periparturient (Table 41.1). Determining when gestational losses occur is the first step in understanding the etiology of these losses and represents the starting point for building the vaccination program.

Utilizing diagnostic tools in cases of abortion can be very helpful and can be very frustrating due to diagnostic limitations and challenges. In cases of abortion, an etiologic diagnosis is identified less than 50% of the time [3]. However, several diagnostic advances, through conscious efforts by diagnostic laboratories, have been made to improve detection of certain pathogens [4]. Even though an etiologic diagnosis can be challenging, infectious agents causing abortions can be identified. Table 41.2 highlights the multiple pathogens that have been associated with bovine abortions, the gestational period they normally affect, and common diagnostic procedures used to identify their presence [2, 5–9]. Some pathogens are considered common reproductive agents and some are rarely identified. In addition, certain pathogens can be more prevalent in certain geographical areas [3].

Diagnostic laboratories have different capabilities in handling an abortion case. As a practitioner, it is prudent to have a close working relationship with your laboratory. Diagnostician consultations are sometimes necessary to develop a systematic approach for abortions. Appropriate tissues and bodily fluids samples are more useful than others are and comprehensive submissions are valuable.

Adequate information, including herd history, with complete submission to a diagnostic laboratory is the most important step to a definitive diagnosis [4]. An abortion work-up can be costly and sometimes unrewarding. It is in the best interest to provide the diagnostic laboratory with the required information to minimize cost and maximize diagnostic success. Even though infectious agents comprise

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 41.1 Pathogens associated with reproductive wastage in beef cows by gestational period.

	Gestational period			
Pathogen	**Early**	**Mid**	**Late**	**Periparturient**
Histophilus somni	Low		High	Moderate
Brucella abortus	Low		High	Low
IBR virus	Low	Moderate	High	Low
Bluetongue virus	Low		Moderate	Low
BVDV	Low	High	Low	Low
Leptospiral serovars	Low		High	Low
Campylobacter fetus subsp. *venerealis*	High	Moderate		
Tritrichomonas foetus	High	Low		
Aspergillus fumigatus			Low	
Ureaplasma spp.	Low			
Listeria monocytogenes			Low	
Chlamydia psittaci			Low	
Parainfluenza 3 virus			Low	Low
Mixed bacteria			Low	Low

Source: From [2], © 1988, Food and Agriculture Organization (FAO).

Table 41.2 Agent-related abortions categorized by period of gestation and diagnostic procedures.

Pathogen	**Gestational period**	**Common diagnostic procedures**
Viral		
IBR	Mid to late	PCR, IHC, FA, VI
BVDV	Mid	PCR, IHC, FA, VI,
Bluetongue virus	Late	PCR, VI
Bacterial		
Brucella abortus	Mid to late	Bacterial culture
Leptospira spp.	Late	IHC, PCR
Campylobacter fetus subsp. *venerealis*	Any	Culture, PCR, MAT, IHC
Ureaplasma spp.	Late	Culture, PCR
Sporadic/opportunistic bacteria[a]	Any	Bacterial culture
Listeria	Late	Bacterial culture, IHC
Chlamydophila spp.	Late	PCR, IHC
Protozoal		
Tritrichomonas	Early	Culture, H + E, PCR, silver stain
Neospora spp.	Mid to late	IHC, PCR
Sarcocystis spp.	Mid to late	IHC
Mycotic/fungal		
Aspergillus fumigatus	Any	Fungal culture, H + E
Mucor/Candida/Rhizopus spp.[b]	Any	Fungal culture, H + E
Unknown agent		
Epizootic[c]/enzootic bovine abortion	Late	IHC, silver stain, PCR

FA, Fluorescent antibody; IHC, immunohistochemistry; VI, virus isolation; PCR, polymerase chain reaction; H + E, hematoxylin and eosin; MAT, microagglutination test.

[a] *Arcanobacterium pyogenes*, *Bacillus* spp., *Escherichia coli*, *Mannheimia haemolytica*, *Streptococcus* spp., *Pasteurella multocida*, *Salmonella* spp., etc.

[b] Many genus and species are associated with bovine abortions.

[c] *Pajaroellobacter aortibovis*.

most of the reported etiology, practitioners, producers, and diagnosticians have to be aware that abortions are not limited to infectious agents [9]. Non-infectious causes, such as environment, toxins, nutrition, and genetics, have to be considered as possible sources of reproductive failures.

Serologic sampling can be utilized in diagnostic work-ups for abortions, but the results should always be interpreted with caution. Single serum samples have little to no value when diagnosing causes for abortions. It is difficult to differentiate the titer values based on vaccine and natural exposure to certain agents/antigens. However, a lack of titer in abortions may rule out certain causes.

Paired serum samples have also demonstrated limited value. Many of the bacterial and viral pathogens that cause abortion may infect the fetus or placenta long before the abortive event occurs. This lag time between infection and abortion may prevent the practitioner from detecting the rising or falling titers associated with the initial infection. This leads to the collection of two "convalescent" serum samples that will fail to detect the increase in antibody titer, if it indeed occurred. This is especially true when only affected females are sampled at the time when the abortion is noted. Overall, the time of seroconversion is dependent on the exposure of the agent and the amount of immunity established prior to the breeding and throughout gestation. Paired sera are much more useful when used as part of a complete diagnostic work-up that includes samples from the placenta, fetus, and fetal fluids.

Serologic profiling is one option to optimize the use of serologic testing. The basis of serologic profiling is analyzing titers from affected/aborted and non-affected dams over the same time period [4]. It is unclear how many samples are needed, but some suggest that the same number of affected and non-affected animals, preferably at same stage of gestation and age, is adequate [5]. In herds with chronic gestational losses, serum may be collected and frozen from a statistically relevant number of cows for future testing as needed. These frozen samples may be collected as the females are processed prior to breeding and/or at the time of pregnancy examination. Then, as fetal loss is detected, banked serum samples can be submitted along with acute and convalescent samples to provide a clearer serologic picture of the affected animals and their normal cohorts. This should give a more complete picture of when seroconversion occurred and what pathogens were involved.

In some cases, all samples will have elevated titers due to endemic infections of specific agents. For example, in herds endemically infected with bovine viral diarrhea virus (BVDV), all animals may have seroconverted yet have no clinical evidence of etiologic diagnosis of abortions [10]. In some instances, fetal/precolostral serology may be beneficial. Fetuses must be immunocompetent for specific agents (*Toxoplasma gondii*/*Neospora caninum*/BVDV/infectious bovine rhinotracheitis [IBR]/*Brucella*) to produce serologic evidence in fetal fluids [3, 4, 11]. However, in some cases of premature placental separation, maternal serum antibodies may "leak" into the fetal circulation and give a false positive result on serology. While serology may be valuable in certain circumstances, the interpretation of these results should be carefully assessed as it relates to the entire diagnostic work-up and clinical signs in the herd.

Designing Herd Vaccination Protocols

While vaccines represent an important tool in protecting reproductive performance, they tend to be somewhat underutilized in beef herds (Table 41.3). When designing protocols to immunize the beef breeding herd against reproductive pathogens, there are several other important factors to consider. The potential at-risk level of the herd should be considered not only from the entry of potential pathogens, but also from the standpoint of the current disease level in the resident herd, different management groups on the ranch, breeding animal movement, and the potential side

Table 41.3 Use of reproductive vaccines by cow/calf operations.

Type of vaccine	Use in weaned heifers (%)	Use in pregnant heifers (%)	Use in cows (%)
IBR	19.4	11.9	24.6
BVDV	25.1	13.7	28.1
Campylobacter	12.6	10	19
Leptospira	19.9	15.1	37.1
Brucella abortus	14.8	2.8	1
Histophilus somni	9.3	5.3	7.9
Neospora	–	0.4	0.3
Tritrichomonas	0.7	0.9	1

Source: Based on [12].

effects of the immunizing agents [2]. While complete protection against every pathogen in every individual is not realistic, the goal would be to minimize the number of susceptible animals in the population. This should prevent epidemic outbreaks of reproductive disease as well as the establishment of chronic endemic losses in the cow herd.

While veterinarians and producers often think of individual vaccination protocols for different management groups on the ranch, it is the author's belief that vaccination programs should be viewed as a continuum. For example, if producers are developing their own replacement heifers, the suckling calf vaccination program should be viewed beyond the summer grazing season and fall weaning events. This vaccination program should be constructed to take into account the probability that these young heifer calves will join the replacement pool, become pregnant, and eventually become a productive member of the mature herd. The suckling calf protocol should be designed to prepare the calf for post-weaning disease challenges and increase the calf's response to subsequent reproductive vaccination. Research has clearly shown that calves vaccinated at an early age will mount a cell-mediated immune response that will enhance the calf's ability to respond to subsequent vaccination or disease challenge [13, 14]. This approach will maximize protection against reproductive pathogens and minimize the potential for any negative vaccine side effects associated with the prebreeding vaccination of seronegative females [15–17]. These side effects may include multifocal areas of ovarian necrosis, hemorrhage and inflammatory cell infiltrate in the ovary, and the development of cysts in the corpus luteum. These lesions are transitory in nature but can result in decreased reproductive performance in the short term.

Other factors to consider in vaccine selection include fetal protection and duration of immunity. Recent advances in vaccine technology and diagnostic testing have allowed vaccine manufacturers to document the ability of their products to prevent disease organisms from spreading to the placenta and fetus following maternal infection. Challenge studies using virulent BVDV, IBR, and *Leptospira borgpetersenii* (serovar *hardjo*) have shown that fetal protection against pregnancy wastage, BVDV persistent infection (PI), and leptospiral renal colonization and urine shedding are possible following vaccination [18–22]. While both modified-live viral and killed virus vaccines have demonstrated fetal protection against BVDV, typically modified-live vaccines provide better protection and a longer duration of immunity [23–25]. Studies have also shown that this protection can last for one year or longer following vaccination of animals of various ages [18, 20, 26]. The concepts of fetal protection and duration of immunity are especially important for beef operations as they are more likely to come in contact with adjacent herds and may only be handled for vaccination once per year.

Before constructing any vaccination program for a cow/calf operation, the potential risk for exposure of the herd to a particular pathogen through herd additions or herd contact with clinical or inapparent carriers of a pathogen should be evaluated. The epidemiological terms "open," "closed," and "modified open" have been used to describe the potential risk level of a given herd [2]. When assessing the need for vaccination, factors such as risk-level management, the magnitude and etiology of previous reproductive losses, herd working patterns and animal management, and the producer's long-term goals should all be considered. Once this information is collected and evaluated, recommendations concerning the use of specific vaccine antigens, the type of vaccine needed, and the frequency of vaccination can be constructed to fit within the confines of the total ranch management plan [2].

First Level: Closed Herd Vaccination

Closed herds run a relatively low risk of disease introduction due to the fact that there is limited movement of breeding animals on and off the operation. These operations develop their own replacement females and have little to no contact with adjacent herds. The primary potential source for disease introduction would be in the form of venereal disease from the addition of breeding bulls. There is also the potential of exposure to leptospiral serovars from domestic livestock and wildlife populations in the area. This type of management will create a herd that is highly susceptible to viral and bacterial reproductive diseases but does so at a very low level of risk for disease introduction [2].

Adequate quarantine protocols and facilities are important for all operations, but this is especially true for closed herds. While the risk of introduction of viral reproductive pathogens is very low in these herds, it can be devastating if it occurs. Therefore quarantine procedures must prevent nose-to-nose contact between new arrivals and the resident cow herd for a minimum of 30 days. This time period should allow animals that are shedding IBR virus following recrudescence or undergoing acute BVD to clear the viremia and stop shedding prior to introduction with the resident herd [27]. Individual animals can also be vaccinated appropriately as they enter and again as they leave the quarantine pens to ensure that herd immunity is maintained and the resident herd protected. This time frame also ensures that all diagnostic screening performed on arrival, such as testing for BVD-PI or *Tritrichomonas foetus*, will be completed and test results returned to the practitioner prior to the animal being released from quarantine. An additional step to enhance biosecurity would be to screen

culled animals annually to gain insight as to the level of seroconversion that is occurring in the breeding herd [2]. Effective quarantine procedures not only help prevent disease introduction but also will enhance the effectiveness of the vaccination program [28].

Vaccination of closed herds should center on pathogens that are difficult to control through biosecurity measures alone. Annual boosters with multivalent products containing *Leptospira* and *Campylobacter fetus* subsp. *venerealis* should be utilized in all breeding females on the ranch [2]. Depending on the normal working patterns on the ranch and the anticipated timing of disease exposure, these vaccine antigens may be given before breeding prior to bull turnout or in mid gestation at the time of pregnancy examination (Table 41.4). While *C. fetus* vaccination would be most effective if given before breeding, it is commonly given in concert with the *Leptospira* serovars in mid gestation since it is unlikely the herd would be handled an additional time. Herd bull vaccination schedules should match the antigens and timing of the female group that they will be turned in with.

Replacement heifers represent the highest at-risk population for reproductive pathogens, both viral and bacterial [2]. The antigens given to these heifers during the suckling calf and weaning phases should prepare these females to respond adequately to the reproductive vaccines given just prior to the first breeding season. This prebreeding vaccination can be administered 30–45 days prior to breeding as the final selection for the breeding pool takes place (Table 41.4). Since these heifers will have had previous vaccine exposure, are relatively stress-free, and are not pregnant, they make the ideal candidates to utilize modified-live IBR and BVDV vaccines. This will enable the herd to maximize protection and minimize potential vaccine side effects [2, 17].

Second Level: Modified Open Herd Vaccination

Modified open herds represent a moderate risk level for disease introduction and should be considered partially susceptible to viral diseases at all times. These herds are best described as those in which the addition of new animals takes place on a limited basis (herd expansion or replacement with purchased additions), or individual animals move in and out of the herd (to livestock shows or exhibitions), or there may be adult animal-to-animal contact with adjoining herds [2]. It is imperative that newly introduced replacements, whether raised or purchased, carry a high level of protective immunity to the most common viral pathogens. Addition of replacements of this type will lessen the need to rely on annual viral vaccination of the resident breeding herd. Well-designed quarantine protocols are important for modified open herds as well.

Table 41.4 Example vaccination program based on risk management concept.

Closed herd
Adult (female and male)
Campylobacter: prebreeding
Leptospira: prebreeding or mid-gestational period
Replacements (prebreeding)
IBR and BVDV: MLV s.c.
Campylobacter: prebreeding
Leptospira: prebreeding or mid-gestational period
Brucella (optional): 4–10 months
Modified open herd
Adult (female and male)
Campylobacter: prebreeding
Leptospira: prebreeding or mid-gestational period
Replacements (non-pregnant, prebreeding)
Brucella: 4–10 months
IBR and BVDV: MLV s.c.
Campylobacter
Leptospira
Replacements (pregnant before introduction)
IBR: killed virus s.c. or i.n.
BVDV: killed virus s.c.
Campylobacter
Leptospira
Open herd
Adult (non-pregnant females, 30–45 days prebreeding)
IBR and BVDV: MLV s.c.
Pregnant females and bulls
IBR: killed virus s.c. or i.n.
BVDV: killed virus s.c.
All adult animals
Campylobacter: prebreeding
Leptospira: prebreeding or mid-gestational period
Replacements (non-pregnant, prebreeding)
Brucella: 4–10 months
IBR and BVDV: MLV s.c.
Campylobacter
Leptospira
Replacements (pregnant before introduction)
IBR: killed virus s.c. or i.n.
BVDV: killed virus s.c.
Campylobacter
Leptospira

Source: From [2], © 1988, Food and Agriculture Organization (FAO).
IBR, Infectious bovine rhinotracheitis; BVDV, bovine viral diarrhea virus; MLV, modified live virus; s.c., subcutaneous; i.n., intranasal.

Yearling replacement heifers represent the main concern for building an immune base within modified open herds. The prebreeding vaccination should include IBR, BVDV, the appropriate *Leptospira* serovars, and *C. fetus* (Table 41.4). These heifers are not pregnant, so it would be preferable to take advantage of the longer duration of immunity provided by modified-live viral vaccines. BVDV types 1 and 2 should be included in the vaccine to ensure a higher level of heterologous protection against strain variation [24, 29]. Whether these heifers are home-raised or purchased, it is important to emphasize the use of vaccines that have been proven to provide fetal protection.

If producers are purchasing pregnant replacements or additions, killed or intranasal combinations of IBR and BVDV provide the best alternative to avoiding the negative side effects of modified-live virus vaccination [2]. This is especially important if the vaccination history of these animals is undocumented prior to their arrival (Table 41.4). Following calving, modified-live viral vaccines may be used before breeding in subsequent years. There are modified-live vaccines that are labeled to be used in pregnant females. However, it is critical that the label directions on these vaccines are followed with regard to the timing and the number of doses given before breeding while the females are not pregnant. This is essential to avoid vaccine-induced pregnancy wastage. As in closed herds, annual vaccination for *Leptospira* and *Campylobacter* is conducted in all adult replacement females and in bulls [2].

Third Level: Open Herd Vaccination

Open herds represent the greatest challenge to a vaccination program since these operations have the highest risk for the introduction of both viral and bacterial reproductive pathogens [2]. These pathogens are typically introduced via animal movement on and off the ranch. This movement typically occurs via the routine purchase of replacements, via direct contact or commingling of the breeding herd with stressed high-risk stocker or feeder calves, or through the sale barn purchase of orphan calves for the purpose of grafting onto a lactating female in the resident herd. These purchased replacements may be heifers or mature cows and can be pregnant or open. The main issue with these purchased females is that the practitioner and producer typically know nothing about their previous vaccination or disease history. Proper quarantine protocols and disease screening at arrival are absolutely critical components to minimize disease risk in this type of herd.

The primary emphasis of the vaccination program for open herds centers on the addition of viral components to maintain a high level of collective herd immunity to these pathogens. This will require annual viral vaccinations in the breeding herd, while continuing annual vaccination against the common bacterial pathogens [2]. A modified-live viral vaccine would be most commonly used 30–45 days prior to the start of the breeding season in open cows. In those situations where the breeding herd is only vaccinated at pregnancy examination (mid gestation) or the herd has a prolonged calving season, the use of an annual killed virus vaccine may be the only option (Table 41.4).

The use of viral vaccines in replacement females should follow the same guidelines as those for the modified open herd. This will minimize the risk of introducing susceptible females into the herd, help establish an immune base for the operation, and ensure that the subsequent pregnancy is protected [2]. Depending on the source of breeding bulls in these herds and the risk of venereal disease introduction, *T. foetus* vaccine could be considered in these heifers and cows. However, a critical review and meta-analysis of the efficacy of *T. foetus* vaccine indicated that while there may be a small positive effect on decreasing abortions in vaccinated heifers, there was no effect on decreasing the infection rate or the risk of being open [30].

The delivery of vaccines for *Leptospira* and *Campylobacter* will be the same in open herds as was described for closed herds (Table 41.4). All adult females, replacement females, and herd bulls will receive these antigens at least annually [2]. These may be given before breeding or at mid gestation, depending on the normal working patterns associated with the ranch management.

Summary

The process of designing immunization programs for beef cattle operations must take into consideration factors such as traffic patterns on and off the ranch, normal handling times, the pregnancy status of the animals to be vaccinated, historical disease patterns, and the relative risk of disease introduction. The goal of the immunization program should be to increase the level of collective herd immunity by minimizing the number of animals that are susceptible to reproductive disease. This not only will prevent epizootic outbreaks of pregnancy wastage, but also should control chronic endemic disease. Our ability to better understand the relationship between reproductive pathogens and the bovine reproductive tract has enabled vaccine manufacturers to provide products that ensure fetal protection and a long duration of immunity, while minimizing negative vaccine side effects. The end result is that the practitioner can provide the client with cost-effective vaccine options to help ensure optimum reproductive performance.

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Dairy Herd Health for Optimal Reproduction

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Introduction

The modern dairy farm is an extremely complex system. Optimal productivity and profitability are dependent upon an interaction of genetics, health, nutrition, reproduction, environment, and management [1, 2]. Good reproductive efficiency, in turn, has a positive impact on animal health. Farm profitability depends upon maintaining dairy cows in the most productive and profitable portion of their lactation curve, returning them there as soon as possible and maximizing the number of lactations in a cow's lifetime [3]. This concept is illustrated in Figure 42.1.

The goal of a modern reproductive herd health program is to maximize reproductive efficiency, that is – to establish a voluntary waiting period (VWP), then to achieve pregnancy as soon as possible after the commencement of the breeding period. Four main factors influence efficiency: the VWP, insemination rate, pregnancy per artificial insemination (AI), and pregnancy loss [4]. The metric best used to evaluate the efficiency of a reproduction program on a dairy farm is the 21-day pregnancy rate (or risk) and is calculated retrospectively:

$$\text{Conception Rate}\left(\text{CR}\right) \times \text{Insemination Rate}\left(\text{IR}\right)$$

This calculation includes only cows eligible to become pregnant once a program is established. Achieving good conception rates depends upon a healthy, properly involuted reproductive tract, a cow in positive energy balance, a fertile ovum, and good AI technique (or a fertile bull). Insemination rate (also referred to as heat detection rate or AI submission rate in some sources) depends upon good heat expression and detection. Many reproduction programs utilize hormonal manipulation of the estrus cycle to synchronize ovulation and insemination, thereby eliminating heat detection as a variable. In this circumstance, insemination rate depends upon excellent compliance with the breeding management program. This simplified description of a reproductive herd health program illustrates the complexity of interactions on a dairy farm where reproduction is involved. It is the role of the reproductive herd health program to monitor and manage these variables to maximize reproductive efficiency. Due to the relationship of biological and management factors that determine when cows become pregnant following the end of the VWP and whether or not pregnancy is maintained, a reproductive program needs to integrate the principles of theriogenology and herd health in order to optimize reproductive performance. This chapter gives an overview of certain health events on a dairy, how they impact reproduction on a herd level, and the development and implementation of herd health programs.

Ketosis and Diseases of Transition

Ketosis, defined as an elevated level of ketone bodies (e.g. betahydroxybuterate [BHBA]) in the bloodstream, occurs when the cow is unable to adapt adequately to a state of negative energy balance [5]. Cows are most at risk during the periparturient period when dry matter intake is low and energy demands from a rapidly growing fetus prepartum or increasing milk production postpartum require body energy stores to be utilized, increasing body condition loss. Ketosis is classified as clinical when characterized by decreased milk production, inappetence, and unthriftiness. Identification of subclinical ketosis (SCK) occurs by measuring an increase in blood ketone (primarily BHBA) levels without observed clinical signs.

The effect of ketosis on individual animal reproductive performance has been demonstrated [5]. Cows that exhibit SCK

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

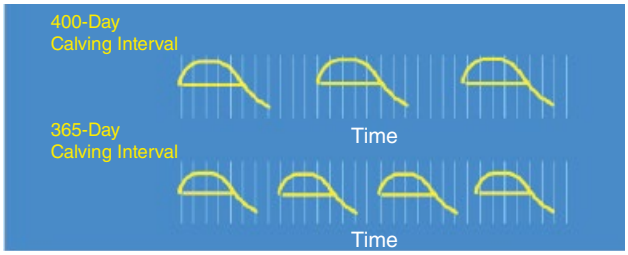


Figure 42.1 Effect of decreasing calving interval on a dairy cow's lifetime milk production and profitability.

early in lactation (less than day 16 postpartum) have reduced fertility at first service and were less likely to become pregnant by 150 days in milk [6]. Studies by Walsh et al. identified a threshold for herd levels of ketosis that affect clinical disease, production, and reproduction. In one study, the median time to pregnancy for cows above the threshold for SCK (BHBA ≥1000 mmol/l in week 1 and ≥1400 mmol/l in week 2 postpartum) was 130 days. Median days to pregnancy was 108 days in cows that never experienced SCK. In addition, first insemination pregnancy rate/AI was reduced by 2% in cows that were ketotic in week 1 postpartum and 3% for those ketotic in week 2 for each 100 mmol/l increase in BHBA [7]. Another study established a herd alarm level for elevated BHBA or non-esterified fatty acids (NEFA). Herd pregnancy rate was found to be reduced by 1.2% in herds when 15% of prepartum animals were found with NEFA ≥0.27 mEq/l and was reduced 0.8% in herds where 15% of prepartum animals had BHBA ≥12 mg/dl. Herd pregnancy rate was reduced 0.9% in herds where ≥15% of postpartum animals had BHBA ≥12 mg/dl [6]. In addition, clinical and subclinical ketosis have a significant downstream effect on transition diseases that can affect reproductive efficiency such as metritis, endometritis, mastitis, immune suppression, and displaced abomasum [6, 8]. Hypocalcemia can also predispose periparturient animals to immune suppression and has been associated with increased incidence of retained placenta and metritis. Hypocalcemia can also lead to increased days to cyclicity and, potentially, increased days open [9]. Managing intake, mineral status, and energy balance through strategies such as proper ration balancing, improved feedbunk access, minimizing pen moves, and reduction of overcrowding are critical to maintaining body condition and achieving maximum reproductive efficiency [8].

Mastitis

Mastitis occurs when inflammatory changes occur in the bovine udder, most commonly because of an invasion by an infectious bacterial organism. Mastitis may be defined as clinical (changes in the character of the milk and/or udder with or without systemic signs) or subclinical (elevated somatic cell counts above 150 000 cells/ml without a change in milk character) [10]. Mastitis is the most common and costly infectious disease of dairy cattle according to survey data of dairy producers [11]. Although estimates of the cost of mastitis on a case basis include the value of herd removals resulting in lost pregnancies [12] or assumptions of an effect on pregnancy rate [13], there is little research to demonstrate the effect of the disease on herd-level reproductive efficiency. However, we can infer its effect on the herd using studies evaluating the influence on individual animal reproductive performance.

Most studies of the effect of mastitis on fertility compare a clinical mastitis event with reproductive performance. Indeed, Hudson and his group found that a clinical mastitis event shortly before or after breeding has a significant effect on fertility [14]. Fuenzalida et al. found that in cows with chronic clinical mastitis (that is, clinical mastitis occurring prior to insemination and continuing through the period of breeding risk), clinical mastitis (disease occurring anytime during the breeding risk period), and subclinical mastitis, the odds of pregnancy are 0.56, 0.67, and 0.75, respectively, compared to cows without mastitis [10]. The period of breeding risk was defined as 3 days prior to 32 days post insemination. Subclinical mastitis seems to have variable results on fertility. Peake did not find a negative effect, whereas Hudson found that subclinical mastitis had a significant effect on the likelihood of an insemination to be successful. His work showed that the odds of a service leading to a pregnancy were decreased by around 18% where a cow exhibited a somatic cell count (SCC) of between 200 000 and 399 000 cells/ml within 32 days of insemination and the odds were decreased by almost 26% when the animal's SCC was >399 000 cells/ml during the same time period [15]. A high proportion of clinical and subclinical mastitis occurs during the first two months of lactation, which overlaps with the interval most producers select as their VWP. It is imperative that producers and herd health veterinarians focus on mastitis control during this period to maximize fertility.

Lameness

Lameness is among the most common [11] and costly [16] diseases of dairy cattle, affecting welfare, productivity, and reproductive performance [17]. Although the diagnosis of lameness is somewhat subjective and determining the severity of the disease depends upon observer bias, the majority of studies show that lame cows resume postpartum cyclic activity more slowly and, once cycling, have poor estrus expression and reduced fertility. Peake and colleagues

found that lame cows diagnosed by a herd veterinarian had 6 more days to the first luteal phase and 9 more days open than herdmates that were not lame. In the same study, cows identified as clinically lame by producers had 6 more days to first service and 43 more days open than their sound herdmates [15]. This study also revealed that lameness in combination with other stressors such as body condition loss and increased SCC have a synergistic effect on the time to an animal's first luteal phase. Morris and colleagues in a UK study supported these findings. This group found that 50% of lame cows either did not ovulate or did not respond to hormonal ovarian stimulation at all during the postpartum period (30–80 days post calving) [18]. Delayed cyclicity and subfertile animals affect herd pregnancy rate during the critical breeding period, negatively impacting herd economics and reproductive success [19].

Heat Stress

Another stressor affecting both expression of estrous and fertility is heat stress. Heat stress occurs when external environmental forces act upon an animal and generate an increase in body temperature enough to evoke a physiological response [20]. Heat stress on livestock is normally measured using the temperature humidity index (THI) which is a calculated value incorporating the combined effects of environmental temperature and relative humidity [21]. A THI of 72 is most commonly used as the upper limit of a diary cow's thermoneutral zone [20, 22, 23], but studies range from 70 to 75 [21]. THI is applied most accurately as a daily average rather than as a measurement at any point in time.

Heat stress causes negative energy balance through reduced feed intake and unique metabolic adaptations, affecting reproductive performance in a similar manner to negative energy balance during the periparturient period [23]. Direct effects on the reproductive tract also affect fertility. Heat stress can affect the reproductive performance of both males and females [22]. It reduces the duration and intensity of estrus behavior [20, 22], and ovulations without estrus signs can occur [24]. Heat may cause physical lethargy and decreased dry matter intake, leading to altered hormone production and thus estrus behavior [20]. Studies have identified effects on fertility such as impaired oocyte quality and embryo development, increased embryonic mortality, endometrial dysfunction, impaired steroidogenesis in ovarian follicles and corpora lutea, loss of normal follicular dominance patterns, and reduced uterine blood flow [25–27]. Schüller [28] showed that even in temperate climates, cows were 61% less likely to become pregnant if a cow was exposed to a THI of ≥73 from 21 days to 1 day prior to breeding. Morton et al. demonstrated that the effects of heat stress on reproduction can be cumulative, particularly from day 35 prior to service to day 6 after service [25]. There is some evidence that embryo technologies such as embryo transfer may have the potential to improve fertility since fertilization and early embryo development are bypassed, which are stages of increased thermosensitivity [4]. These data show that producers must employ heat stress mitigation throughout the hot season even in temperate climates to maximize reproductive performance.

Prevention of Infectious Disease

The effects of infectious viruses and bacteria on the health and reproductive performance of dairy cows are well covered in other parts of this text. The role of the herd health advisor should be to design an effective biosecurity and vaccination program to maintain the health and productivity of the herd. When designing an effective vaccination protocol, this author employs a system similar to that designed by James S. Cullor and Anne M. Nelson at the University of California in the 1990s called Dairy Breakthrough Management (BTM). This system, influenced by the US Food and Drug Administration (FDA)'s hazard analysis critical control point (HACCP) program and other management materials, uses a 12-step approach to design protocols that are consistent and workable, and that ensure buy-in by all members of the management team on a dairy.

BTM and similar systems are designed to anticipate an outcome and identify those steps where control can be applied and that are essential to prevent the outcome being undesirable (for example, disease, abortion, or infertility due to an infectious disease). These steps, called *critical control points*, are assigned monitoring procedures, critical limits, and corrective actions if the identified critical limits are exceeded.

In general, using this approach, the process to design an effective vaccination protocol may look like this:

1) Perform a hazard analysis
   a) What is the goal of the program?
      i) Prevent respiratory, digestive, or other disease?
      ii) Protection of conception?
      iii) Fetal protection?
   b) What is the current program?
      i) Is compliance adequate?
      ii) Is it accomplishing the stated goal or goals?
   c) To what disease risks are the animals exposed?
      i) What is the current state of disease on the dairy?
      ii) Are animals of unknown disease status being brought onto the farm?
      iii) Is natural breeding employed?

   d) Are there management factors or other stressors that may affect response to the program?
      i) Is nutrition adequate?
      ii) Is stocking density appropriate?
      iii) Is concurrent stress from lameness or other factors present?
2) Assemble a management team to design the new program
   a) Ownership or upper management
   b) Herdsman
   c) Veterinarian
   d) Anyone who will be handling or administering the vaccines
3) Identify the desired outcome
4) Discuss the current work flow situation
5) Identify critical control points
   a) Examples:
      i) Is the vaccine properly stored, mixed properly if required, and promptly administered?
      ii) Is there an adequate inventory management program in place so the farm does not run out?
      iii) When are animals handled?
      iv) How many vaccines are in the protocol? How many will be given concurrently?
      v) Is the right product going to the right animals?
      vi) Is the vaccine administered by the appropriate route?
      vii) If boosters are required, are they being given correctly?
6) Write the protocol
7) Decide what should be monitored and set action points
   a) Is disease incidence under control?
   b) When to reorder if inventory gets low
   c) Does the inventory match the number of doses that should have been administered?
8) Develop a record keeping system.

Although the system described here is for the development and implementation of a vaccination protocol, the same approach may be useful for development of other treatment protocols, management protocols (such as a managed breeding protocol), and standard operating procedures on a dairy farm.

Developing and Implementing Reproductive Herd Health Programs

The definition of a reproductive herd health program has changed significantly since the 1960s when producers and veterinarians became more proactive in their approach to reproduction. During this period and throughout the 1970s, veterinarians and dairy producers developed routine scheduled visits designed to identify non-pregnant animals and re-enroll them into a reproductive management program. The advent in the 1990s of hormonal manipulation of the estrus cycle, synchronization protocols, and advanced reproduction and production data management programs facilitated the evolution of modern reproductive herd health programs. During this time, veterinarians coined the term "production medicine" to recognize the interrelationships health, reproduction, and management have on dairy farm profit. Today's production medicine veterinarian employs a holistic view of the dairy farm – incorporating nutrition, health maintenance, reproduction management, genetics and genomics, youngstock management, economics, employee training, and protocol development. Each area of this complicated system must be monitored and managed to maximize production, reproduction, and farm profit.

In the latest National Animal Health Monitoring System (NAHMS) dairy management survey, over 85% of dairy farms surveyed used veterinary services for reproductive management [11]. Reproductive efficiency has a major impact on farm profit, animal health, and replacement inventory [3]. Shortening the calving interval reduces the average days in milk for the herd, resulting in more cows in the early, more profitable part of their lactations [4]. The cost of a day open increases with greater calving interval, with estimates ranging from USD $1.25 at 90 days to $2.75 at 201 days in milk. It can vary, however, from $0 to $6.00 based on a variety of factors [4]. Shorter lactations also promote animal health and welfare by maintaining animals in more optimum body condition. Progressive dairy producers will challenge veterinarians to design a reproductive herd health program that meets the farm's objectives in these key areas.

Setting Goals

A wise colleague once told this author that he made much more progress in his herd health programs once he stopped telling producers what to do and began asking them about their goals (W.M. Hilton, personal communication). For veterinary recommendations to be successful, advice should meet three criteria [29]:

1) Advice should be based on scientific evidence.
2) Advice should be effectively communicated.
3) Advice should build on the goals of the producer in order to take advantage of their motivation to implement change.

Unfortunately, too often the goals of dairy farmers and their advisors do not match. In a study from the Netherlands, 16 of 22 farmer : veterinarian combinations did not match in their primary goal for the farm. In general, veterinarians overemphasized production and udder

health and farmers more often prioritized cow fertility [29]. Goal setting is an integral part of modern management programs like the previously mentioned BTM. Dairy farmers and their advisors should routinely have a frank conversation about farm goals to ensure that each understands the priorities that the producer has for the farm's herd health and reproduction programs.

Determining the Ideal VWP

The first component of herd reproductive efficiency is the establishment of an appropriate VWP. The VWP is defined as the time a dairy farmer is willing to wait after parturition before cows are eligible for insemination, normally reported in days [30]. The goal of the VWP is to improve conception rate at first service by allowing time for uterine involution and clearance, improved energy balance, and the return of normal cyclicity [4]. The ideal VWP for an individual herd is partly determined by reproductive efficiency represented by the herd pregnancy rate and partly by the anticipated optimum time for the establishment of pregnancy [3, 4]. In general, cows with lower milk production, poor lactation curve persistency, and a lower 21-day pregnancy rate require a shorter VWP, while herds with higher producing cows, persistent lactation curves, and a high 21-day pregnancy rate could benefit from a longer VWP [3, 4].

In an economic analysis, Ribeiro and colleagues looked at two periods (110 and 130 days) for optimum days to pregnancy after calving. They found that in herds with poor 21-day pregnancy rates (<15%) these goals could not be met, even if breeding commenced immediately after calving. In herds with 21-day pregnancy rates >22.5% the goals could be met with VWP of between 60 and 98 days [4]. The most recent averages for >9800 dairy herds on Dairy Herd Improvement Association (DHIA) records from the USA that are processed at the Raleigh, NC testing center were for an average VWP of 59.4 days and a 21-day pregnancy rate of 19.2%. Average days to pregnancy was 148.9 days (DairyMetrics, Dairy Records Management Systems [DRMS], Raleigh, NC). One should also consider individual cow factors such as parity, milk production, and persistency of lactation when establishing the length of the VWP. A longer VWP does not necessarily result in a longer calving interval if conception rate to first AI is good [4].

Strategies to Increase Insemination Rate

The second component of reproductive efficiency is to maximize insemination rate once the VWP has passed. Insemination rate is dependent upon cows in estrous being delivered for breeding. Traditionally this was accomplished by observing estrus behavior, and this is still the system used by just over half of dairy producers for first service [11]. However, whether or not cows are inseminated in this system is based on the efficiency of estrous detection, which is low in most herds. External factors such as increasing heat stress, herd size, animal density, and confinement housing on concrete, as well as individual animal factors discussed previously can affect estrus expression [4]. The majority of dairy farms are now using technologies such as timed AI (TAI) programs and activity monitors to increase insemination rate at some point during the breeding period [11].

The advent of luteolysis and subsequent ovulation from the use of exogenous prostaglandins in the 1970s improved reproduction management in dairy cattle, although timing of estrus expression using this system is variable dependent upon external factors and individual animal status [4]. The development of TAI programs in the 1990s further improved insemination rates by synchronizing ovulation and insemination. These programs are particularly effective in herds where heat detection is a challenge and they provide a significant economic benefit over heat detection alone [4]. Designed initially as programs intended to increase insemination rates, newer methods of TAI incorporate knowledge about bovine reproductive biology, hormonal manipulation, and oocyte and embryo quality to improve conception and pregnancy retention [4].

Among the dizzying array of TAI program options, herds that employ a strategy of TAI for first insemination (no matter which specific protocol) coupled with estrus detection experience the lowest median days to pregnancy and more profit per cow [31]. In a simulation evaluating reproductive performance and economics of 10 different breeding programs by Galvão et al., the highest 21-day cycle pregnancy rate was obtained when cows were subjected to a TAI program with 95% compliance of treatments for first insemination followed by estrus detection at 60% efficiency with 95% accuracy. It was demonstrated that the greatest improvement in economic return in the simulation was in those herds that previously had poor compliance of treatments [31]. It follows that optimal reproductive efficiency and herd economics occurs in herds that incorporate an effective and compliant TAI protocol for first service followed by estrus detection, and that efficient and accurate detection of estrus activity remains an integral part of successful dairy reproductive performance.

Automated estrus detection systems are increasingly in use by dairy producers [11]. These systems generally work by measuring a cow's activity via a neck-mounted activity meter or a leg band with a pedometer and generating a notification when an increase in activity exceeds a designated threshold as determined by computer algorithms [32]. Studies have indicated that automated systems can identify

as many as 80% of cows in estrous accurately [32, 33]. Limitations on this technology include multiparous cows, lame cows, cows with low body condition score (BCS), and cows with purulent vaginal discharge. These animals tend to exhibit poor activity [32]. Sensitivity of detection can be influenced by such activities as being on pasture, pen moves, hoof trimming, and other management events that may increase measured activity. Although economic analyses do not necessarily show an economic advantage to these systems [34], automated systems will likely become more common in an industry with progressively restricted access to pharmaceuticals.

As recently as 2014, a NAHMS survey [11] found that over half of dairy operations in the USA incorporated natural service with bulls somewhere in their breeding program and 10% used bulls exclusively [11]. Days to pregnancy and 21-day pregnancy rate may be improved in bull-bred herds over herds that employ AI [35]. However, a 2010 economic analysis found that cows in herds enrolled in a TAI program enjoyed a benefit of nearly $38.00 per pregnancy over cows in herds that employed natural service when pregnancy rate and VWP were standardized. This advantage depended on the cost to feed bulls, semen price, and genetic merit of semen [35]. Natural service may be an option for those producers who lack the management ability, resources, or labor to manage an effective AI program, but it is not the most effective strategy to maximize reproductive efficiency, genetic advancement, or economic performance of dairy herds [3].

Managing and Monitoring Fertilization Failure and Pregnancy Loss

Recognizing and monitoring pregnancy per AI and pregnancy loss is a vital component of reproductive efficiency. A review article by Wiltbank and colleagues summarizes studies involving pregnancy loss outside of that caused by infectious disease [27]. The authors identified four distinct periods of risk and described the herd health effects that influence the loss of the conceptus during these periods.

Period one includes fertilization and the first week of pregnancy. In thermoneutral conditions in healthy cows with high-fertility semen, fertilization failure occurs in 10–20% of inseminations. Factors involved in increased fertilization failure include improper AI technique, inflammation and the presence of inflammatory cells in the uterus, poor quality semen, and heat stress. Once fertilization occurs, increased degeneration of embryos in the first week can occur because of the ovulation of larger, possibly persistent follicles due to inadequate circulating P4 during follicular development. One theory holds that these larger follicles may be a response to increased body condition loss [36]. One way synchronization protocols can increase embryo quality and survivability is by ensuring that smaller, less persistent follicles are presented for fertilization [27].

The second critical period occurs from day 8 to 27 and may result in 10–50% of pregnancy losses. Three important factors occur during this period. The first factor is the growth of the embryo and development of the trophoblastic tissues. This factor may again be associated with low circulating P4 levels. Nutritional support of the dam is vital; interestingly, three amino acids identified as limiting for milk production (lysine, methionine, and histidine) are those that exhibit the greatest increase in the uterine environment during embryo elongation. In addition, body condition loss can affect P4 levels, as described previously. The second factor is maternal recognition of pregnancy. Occurring at day 16 in the bovine, maternal recognition is especially dependent upon proper embryonic development, as described above. The third factor is the suppression of the maternal immune system, requiring a healthy maternal immune system and immunological diversity. Heat stress can also affect embryonic degeneration during this critical period.

Period 3 is days 28–60 of gestation. This period may account for around 12% of pregnancy losses. This period is especially important to monitor for reproductive efficiency as evaluation for pregnancy normally occurs around days 28–30, and therefore losses during this period may occur after pregnancy diagnosis. Risks for pregnancy loss during this period include increased body condition loss, anovulation at the end of the VWP, reduced P4 circulation during dominant follicle growth, the presence of uterine disease or non-uterine disease such as mastitis, and nutritional factors such as vitamin E/selenium deficiency.

Critical period 4 occurs from day 60 to day 90 after insemination. This period accounts for 1–3% of pregnancy losses. Up to 75% of losses during this period may be due to twin pregnancies, especially those occurring in the same uterine horn. Proper maternal nutritional support is also critical during this period [27].

Evaluation for Pregnancy Status

Reproductive efficiency depends upon early pregnancy evaluation and, if the cow is not pregnant, re-enrollment into the breeding management program. The ideal test for pregnancy should have a high sensitivity and specificity, be cost effective, simple to perform under field conditions, and supply results at the time of the test [37]. Direct transrectal palpation of the reproductive tract is the oldest and most well-established method. Experienced palpators are highly accurate at 30 days and above after insemination.

Transrectal ultrasound may be used to diagnose pregnancy as early as 26 days, but sensitivity below 30 days is decreased due to pregnancies normally lost after this point. Diagnosis at this early date is more accurate when a fetal heartbeat is visualized [37]. Due to the number of pregnancies that are normally lost after this point, as mentioned above, programs that include pregnancy testing at day 28–30 should include re-evaluation later in gestation.

A commercial test for pregnancy-associated glycoprotein-B has been available since the 1990s for the diagnosis of pregnancy in cattle. The test is highly sensitive, but specificity varies from 83 to 97% depending upon the study and the methodology [37, 38]. With this test, blood samples are submitted for evaluation and results are not normally available cowside. In the future, in-line testing of pregnancy-associated proteins and glycoproteins in milk may supply dairy producers with immediate, day-to-day pregnancy status in their animals [37].

Monitoring Health Effects on Reproduction

Accurate and consistent records are essential for proper management of health and reproduction. According to the 2014 Dairy NAHMS survey, a record-keeping system is in use to track individual animals by 95.0% of all operations and 100.0% of large operation [11]. Many smaller dairy farms cling to a paper-based system, but the majority (94%) of large operations use a computer-based system. As performance and reproduction have advanced through the implementation of synchronization protocols, monitoring compliance and outcomes via records systems have become important metrics in their success (Dr. Mark Kirkpatrick, Zoetis Animal Health, personal communication). A more detailed description of record-keeping systems and records analysis is found in Chapter 45 of this text.

These powerful systems may be employed in the monitoring of the effects of herd and individual animal health on reproductive efficiency. Often, producers take advantage of the flexibility of these programs to define disease conditions or use farm-specific or vague definitions (e.g. "SICK") or conditions based on the farm-specific treatment employed. Disease definitions within records systems should be standardized to enable more effective and consistent monitoring by producers, veterinarians, and advisors. Kirkpatrick [39] argues that disease monitoring be standardized with the definition of nine clinical diseases of economic importance: milk fever, retained placenta, metritis, ketosis, displaced abomasum, lameness, clinical mastitis, pneumonia, and digestive disease. Producers can quantify these conditions' effects on profitability and reproductive efficiency by evaluating:

- The proportion of animals affected with a specific condition removed from the herd by 30 days after onset.
- The proportion of affected individuals dead by 30 days after onset, to judge the lethality or severity of the condition.
- The effect on milk yield associated with the condition.

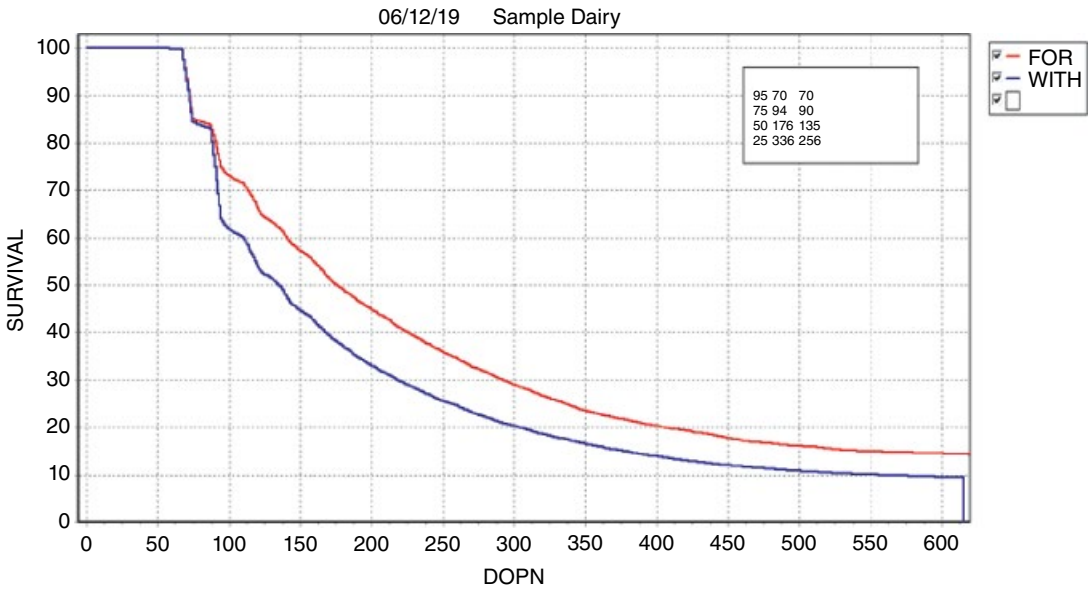


Figure 42.2 Example of a Kaplan–Meier survival curve showing effect of a health condition on days open, where FOR represents the effect a condition (e.g. mastitis) has on days open (DOPN, expressed as days after calving) and WITH represents animals without the condition. The X axis represents risk of survival in the herd. *Source:* Courtesy of Mark Kirkpatrick.

The condition's impact on reproductive efficiency can be further evaluated by determining the proportion of cows remaining in the herd that are pregnant by 120 days in milk. With accurate and consistent record-keeping, additional reproductive insights can be had using tools such as the Kaplan–Meier survival curve analysis in DairyComp 305 (Valley Ag Software) which help to illustrate the effect a condition has on metrics such as days open and survival in the herd [39] (Figure 42.2).

Summary

Dairy herd health and reproductive efficiency are interrelated factors that have a substantial effect on herd productivity and profitability. Dairy producers along with their herd health veterinarian and other advisors should work together to design a reproductive herd health program that recognizes and meets producer goals. The program should identify an economical VWP, then incorporate a protocol that maximizes the number of cows presented for insemination, followed by accurate estrus detection. Metabolic diseases, infectious disease, mastitis, lameness, and heat-related stress should be minimized for optimum fertility. Producers should develop accurate and consistent record-keeping systems to monitor animal health and the consequences on reproduction. Setting achievable goals, implementing programs, identifying bottlenecks to success, and monitoring effectiveness are the hallmarks of a modern production medicine system on a dairy farm.

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Herd Diagnostic Testing Strategies

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Introduction

Veterinary practitioners help food animal clients meet a number of specific herd reproduction goals. These include having a high percentage of females exposed to bulls becoming pregnant (i.e. enhancing fertility or minimizing infertility); minimizing the effects of infectious, metabolic, and other disease processes that can cause pregnancy loss or other disease loss; and enhancing the genetic value of the herd through selection and multiplication of economically superior parent animals. To aid their evaluation of an operation's reproductive efficiency, practitioners have a number of tests available to them for use in screening, monitoring, and diagnosing problems in populations and individuals. Any test or procedure that reduces the uncertainty about a disease or production characteristic of an individual animal or herd can be broadly characterized as a diagnostic test [1, 2]. Commonly utilized diagnostic procedures include use of laboratory tests for infectious disease agents, such as serology, immunohistochemistry, polymerase chain reaction (PCR), virus isolation, and bacterial culture. These tests can be used to screen for asymptomatic infectious diseases in apparently healthy animals, surveillance for persistently infected animals for culling from the herd, exclusion from herd entry, or segregation from susceptible herd mates, and to investigate disease outbreaks when clinical symptoms are not specific for a particular infectious agent. In addition, veterinarians use other types of diagnostic procedures to examine individual livestock and entire herds to find and change avoidable risks, for breeding soundness examination of bulls and heifers, for body condition scoring, to diagnose pregnancy via uterine palpation, ultrasonic examination, or blood metabolites, and for feed and ration evaluation (nutritional and toxicological).

Determining Diagnostic Test Usefulness

A valid question confronting veterinary practitioners is whether to use available diagnostic tests for a specific condition [3, 4]. The input that one needs in order to arrive at a logical conclusion includes: ease of test use and interpretation, prevalence or probability data about the condition or disease, diagnostic sensitivity and specificity data, disease or condition epidemiology, and economic costs of the condition and its treatment or prevention [4, 5]. Literature review and mathematic aids such as computer spreadsheets are the tools used to calculate the post-test predictive values of diagnostic tests, economic value of testing, sensitivity of the decision to the individual inputs, and the importance of individual inputs to the decision. These calculations can then be used to evaluate alternative diagnostic testing strategies and to identify the control points that will be monitored for change that can trigger a reevaluation of the decision.

Sensitivity and Specificity of Diagnostic Tests

When considering the use of diagnostic tests to guide clinical decisions, it is very important to understand that the term sensitivity is used to describe test performance in two different contexts, analytical and diagnostic; and a clinician must think about diagnostic test sensitivity in the correct context or risk being misled by the test results [6]. In exactly the same way, when integrating information provided by a description of test specificity, it is critical to know if the information is describing analytic specificity or diagnostic specificity.

Analytic sensitivity is a laboratory characteristic of the test in that it signifies the smallest amount of substance or

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slightest magnitude of detectable characteristic (visual, olfactory, auditory, etc.) that can accurately be measured from the tested specimen. A test with high analytic sensitivity is able to detect very low concentrations of substance, while a test with low analytic sensitivity would not detect a substance unless the substance was fairly abundant. In contrast, diagnostic sensitivity is a test property that is determined by comparing the test to a "gold standard" test, which is considered to be the true diagnosis as determined using information such as postmortem findings or pathognomonic clinical characteristics or laboratory results. Diagnostic sensitivity is determined in populations of animals and is the percentage of animals that have a particular status (infected, diseased, pregnant, infertile, etc.) that are identified by the diagnostic test as being gold-standard positive for the status.

Analytic specificity denotes the ability of a diagnostic test in a laboratory setting to explicitly classify one particular organism, substance, or status rather than other organisms, substances, or statuses [6]. A test with high analytic specificity would have low cross-reactivity with non-target substances, while a test with low analytic specificity may return a positive result from a specimen that does not have the characteristic or substance targeted by the test because some other characteristic or substance interacts with the test method to return a positive result. In contrast, diagnostic specificity is determined in animal populations and is the percentage of animals that do not have a given condition that are identified by the test as negative for the condition.

Analytic sensitivity is measured as a concentration, such as nanograms per milliliter of serum, parts per million, or particles per microliter, while diagnostic sensitivity is reported as a percentage with the number of true-positive animals in the numerator and the number of animals that truly have the condition in the denominator (includes both the number of animals that have a false-negative classification and the number of animals that have a true-positive classification). Analytic specificity might be reported as the percentage of specimens from animals that do not have the target condition that test negative, while diagnostic specificity is reported as a percentage with the number of true-negative animals in the numerator and the number of animals that are truly free of the condition in the denominator (includes both the number of animals that have a false-positive classification and the number of animals that have a true-negative classification).

Diagnostic testing used in human medicine in the USA is under regulatory oversight whether the test is done in a diagnostic laboratory or a doctor's office, but veterinary medicine is not similarly scrutinized [7]. The initiation of regulatory oversight in human medicine was in response to the recognized importance of rigorous quality assurance and quality control in order to be reasonable assured of providing medical interventions based on accurate diagnostic assessments. Without enforced regulatory assessment of diagnostic tests, veterinarians must recognize the need for quality assurance and quality control and accept the responsibility for frequent and rigorous assessment of the diagnostic sensitivity and specificity of all the diagnostic tests utilized by the practice – not just laboratory tests, but also breeding soundness examination of bulls, reproductive tract scoring of heifers, pregnancy diagnosis, body condition scores, respiratory tract auscultation, and many other commonly implemented clinical procedures.

Diagnostic tests try to separate two populations. One is abnormal, is diseased, or has an undesired condition and the other population is normal or has a desired condition. In most conditions of interest to veterinarians, the affected and unaffected populations overlap based on available diagnostic tests; therefore both laboratory and clinical examination tests must use an arbitrary cutoff to separate test-positive and test-negative populations [2]. Where one places cutoffs for diagnostic tests is very important when deciding into which of two distributions of outcomes a particular animal or herd falls. Because diagnostic measurements of affected and unaffected populations overlap to some extent, diagnostic sensitivity and diagnostic specificity are inversely related, and placing the cutoff is always a trade-off between the impacts of false-negative and false-positive results (Figure 43.1). For test cutoffs not set by the practitioner (i.e. set by a test manufacturer or at a diagnostic laboratory), it is important for the practitioner to be informed of the test's diagnostic sensitivity and specificity, preferably determined in a population that is similar to the population to which the test will be applied.

Where one places a diagnostic cutoff is always a trade-off between false-negative and false-positive results because of the overlap between normal and abnormal populations [8]. Using an example of tachypnea as evidence of respiratory disease in calves requires that the veterinarian define tachypnea with a cutoff number of breaths per minute. Using a cutoff of 25 will likely be more sensitive for detecting calves with pneumonia, but is likely to result in the false-positive classification of many non-pneumonic calves. Placing the cutoff at 40 will increase diagnostic specificity and will reduce the number of non-pneumonic calves classified as tachypneic, but may also fail to identify some truly pneumonic calves (increased false-negative classifications).

Prevalence

Prevalence is the proportion of animals that meet a particular case definition at a given time to the size of the population at that time. The prevalence is the probability of the condition being present in a randomly selected individual from that population. Unfortunately, there is often

(a) In this figure, the cutoff is placed to maximize sensitivity so that there are few false-negative results, but false-positive results occur

(b) In this figure, the cutoff is placed to maximize specificity so that there are few false-positive results, but false-negative results occur

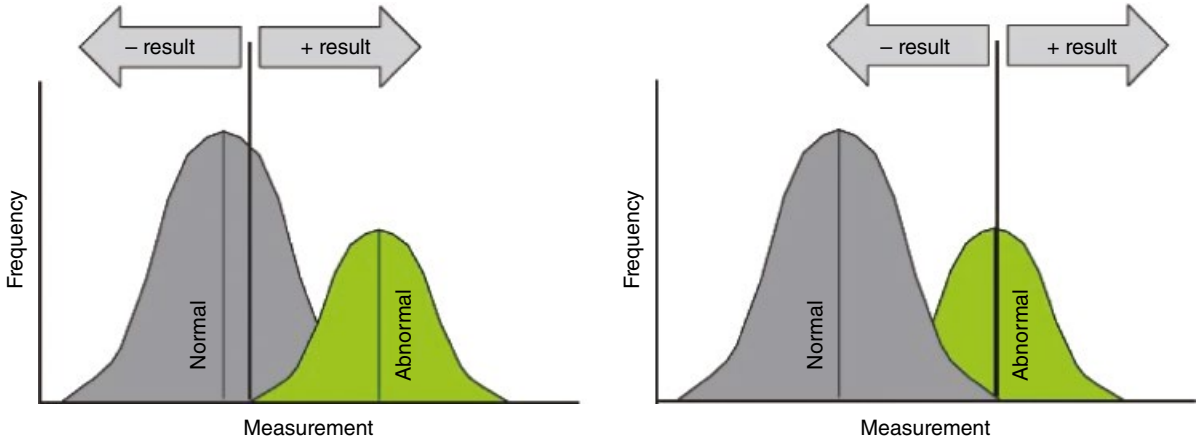


Figure 43.1 When diagnostic measurements of affected and unaffected populations overlap to some extent, diagnostic sensitivity and diagnostic specificity are inversely related, and placing the cutoff is always a trade-off between the impacts of false-negative and false-positive results.

either limited published prevalence information or the published prevalence estimates are so broad as to be of limited usefulness for most of the infectious diseases and reproductive conditions of interest to veterinary medicine. Each practitioner's judgment, based on history and clinical examination of both individuals and the population, aided by what prevalence information is available, is often all that is available to establish the probability for both infectious diseases and reproductive conditions.

Knowing or estimating prevalence is important when interpreting diagnostic tests because for tests with imperfect diagnostic specificity, an increasing proportion of the animals that test positive will be false positives as prevalence or disease probability decreases. For biosecurity reasons, veterinarians often test cattle from very low prevalence populations, so that even a highly accurate test will render inaccurate positive test results when applied to low-risk populations as indicated by the test's low positive predictive value (PPV) in these types of populations.

Similarly, when a test has imperfect diagnostic sensitivity, an increasing proportion of the test-negative animals will be false negatives as prevalence or disease probability increases. Therefore even a highly accurate test can result in inaccurate negative test results (low negative predictive value, NPV) when applied to a population with high prevalence or probability for the condition.

Post-Test Predictive Value

The post-test predictive values of a test are determined not in the laboratory but in the field with the populations where the test is applied; they tell a veterinarian if a valid test is useful in specific populations [2]. The PPV is the proportion of animals with a positive test result that are actually positive for the disease or condition in question and in most situations is influenced more heavily by the test's diagnostic specificity than diagnostic sensitivity. The NPV is the proportion of animals with a negative test result that are truly negative and in most situations is influenced more heavily by the test's diagnostic sensitivity than diagnostic specificity. Both the PPVs and NPVs of a test are affected by the prevalence of the condition in a population (or probability of the condition in individual animals). As the prevalence of the condition is raised, more animals with the condition are present in the population and one has greater confidence that a positive test result is correct. With increasing prevalence, when diagnostic sensitivity and diagnostic specificity are held constant, the PPV of the test is increased and the NPV is decreased, while the reverse is true as the prevalence of the condition is decreasing (Figure 43.2).

The probability that an animal that tests positive is truly positive (PPV) is computed as:

$$\text{Positive predictive value} = (\text{Se} \times \text{P}) / \left\{(\text{Se} \times \text{P}) + \left[(1-\text{P}) \times (1-\text{Sp})\right]\right\}$$

The probability that an animal that tests negative is truly negative (negative predictive value or predictive value of a negative test) is computed as:

$$\text{Negative predictive value} = (1-\text{P}) / \left\{(1-\text{P}) + \left[(1-\text{Se}) / (\text{Sp})\right]\right\}$$

where Se is the test's diagnostic sensitivity, P is prevalence, and Sp is the test's diagnostic specificity.

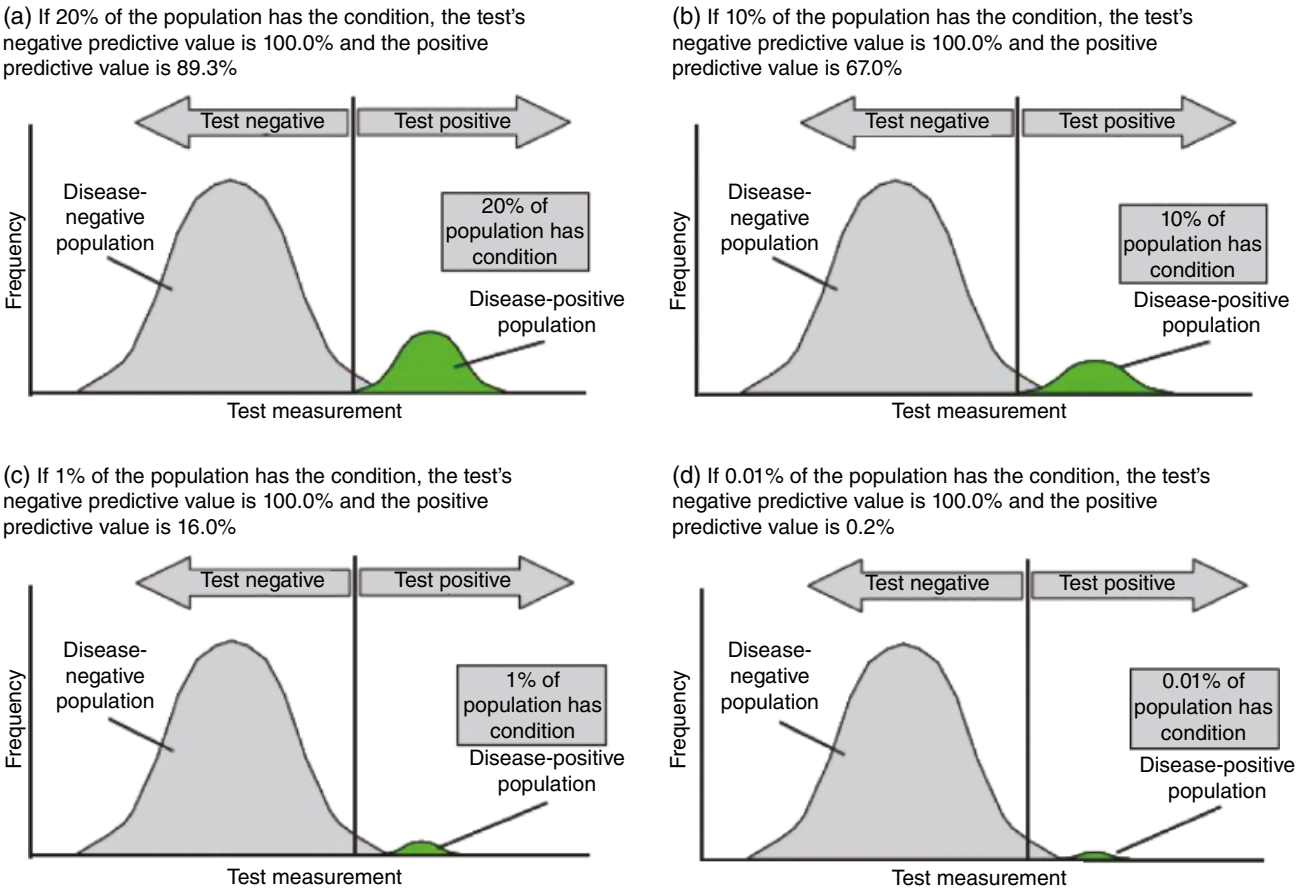


Figure 43.2 If the test has imperfect test diagnostic specificity (i.e. <100%), as the prevalence of the condition decreases, the PPV of a positive test decreases. In these examples, the diagnostic sensitivity and the diagnostic specificity are consistently 99.99 and 95%, respectively, and the prevalence of the condition changes.

An example of how the probability of a condition in certain classes of cattle or in herds with particular previous diagnoses or other history characteristics affects test interpretation is the different certainty a veterinarian will place on a positive test result for bovine viral diarrhea persistent infection (BVD-PI) status depending on the animal signalment and herd history. Using a BVD-PI test that has a diagnostic sensitivity of 99.7% and a diagnostic specificity of 99.3% in different situations will result in different PPVs and hence the certainty of the diagnosis, which will influence the follow-up steps by the veterinarian. If the BVD-PI test is applied to a calf that died following a short disease course of pneumonia and diarrhea in a herd with confirmed cases of BVD in the past two months, the veterinarian may estimate that the probability of the calf being BVD-PI is about 70% before he or she requests the diagnostic test. With a 70% probability of BVD-PI status and diagnostic sensitivity of 99.7% and diagnostic specificity of 99.3%, the PPV is 99.7% and the veterinarian will likely believe a positive test result. In contrast, the veterinarian for a seedstock client who routinely tests all calves on the ranch for BVD-PI status as part of a rigorous biosecurity protocol and as a marketing tool to promote their high-health calves may estimate the probability of finding a truly BVD-PI animal to be very low (<0.1% probability). Using the same tests (99.7% diagnostic sensitivity and 99.3% diagnostic specificity) on this low-risk herd results in a PPV of 12.5% and the veterinarian will likely be very skeptical that the calf is truly BVD-PI.

Diagnostic Testing Strategy

After a single test, it is very unlikely to have both a near-perfect PPV result and a near-perfect predictive value of a negative test result; therefore veterinarians must recognize that they are likely to be placed in the situation where one can only believe one test result direction, either positive or negative. The veterinarian must then decide what to do with the animals that return a test result that is not trusted: either retest the animal or knowingly accept the possibility of a false-positive (situation with low PPV) or a false-negative

(situation with low NPV) without further diagnostic effort. That is, you accept that you may cull or treat some false-positive animals if the cost of further diagnostics is greater than the cost of false-positive intervention; or you accept that you may keep in the herd or fail to treat some false-negative animals if the cost of further diagnostics is greater than the cost of false-negative intervention.

There are two general categories for testing strategies that utilize more than one diagnostic test to determine test-positive or test-negative status. The first of these is described as "testing in series" and is usually used to retest animals with positive test results when the PPV is low after the initial test. The reasons a test would have a low PPV are either low diagnostic specificity of the test or low prevalence in the population. Interpretation of two tests in series requires positive test results in both tests in order to meet the case definition for a positive conclusion. The net results of "testing in series" are a loss of diagnostic sensitivity compared with a single test but a gain in diagnostic specificity. Therefore the PPV is improved and NPV is reduced when interpreting tests in series versus using a single test.

There are several reasons to test in series. In some instances, the second test is more expensive, time-consuming, or difficult than the initial test; therefore we only want to use that test in higher prevalence populations. In other situations, the effect of false positives after a single test is more detrimental than the benefit of finding all instances of disease. If a calf tests positive for BVD-PI status from a herd with low probability of BVD, the PPV will be low and the value of the calf should cause the veterinarian to run a second test interpreted in series and the calf would only be classified as truly BVD-PI if it has a positive result to both the initial test and the follow-up test.

The other category of multiple testing strategies is to test "in parallel" and is usually used to retest animals with negative test results when the predictive value of a negative test is low after the initial test. The reasons that a test would have a low NPV are either that the test has low diagnostic sensitivity or the population has a very high prevalence for the condition.

The current tests for *Tritrichomonas foetus* have very good diagnostic specificity but far less than 100% diagnostic sensitivity, with estimates of 53–77% under field conditions [9–11]. If a veterinarian is testing bulls in a situation where the possibility of trichomoniasis is at least 50%, the NPV is only about 77%, meaning that many test-negative

(a) In this diagram, the test has very good sensitivity and negative predictive value, but the specificity is less than 100% and the positive predictive value is poor. Therefore, the test-positive animals should be re-tested and interpreted "in series".

Positive test results are not trusted:
- Retest positive animals
- Interpret tests in series
- Second test must be positive for the animal to be considered test-positive

(b) In this diagram, the test has very good specificity and positive predictive value, but the sensitivity is less than 100% and the negative predictive value is poor. Therefore, the test-negative animals should be re-tested with a second test interpreted "in parallel".

Negative test results are not trusted:
- Retest negative animals
- Interpret tests in parallel
- Second test must be negative for the animal to be considered test-negative

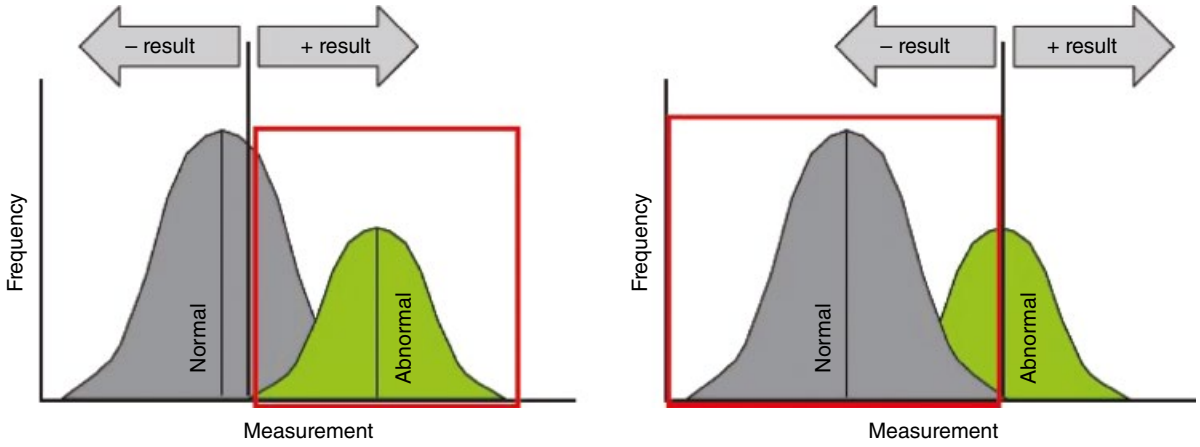


Figure 43.3 After an initial diagnostic test, one test result direction (test-positive or test-negative) should be very good (near-perfect predictive value), but the other test result direction is likely to result in a much lower post-test predictive value. For conditions affecting bovine reproduction, additional diagnostic tests are interpreted "in series" for situations where the veterinarian does not trust initial positive test results and test-positive animals are retested. Additional tests are interpreted "in parallel" for situations where the veterinarian does not trust initial negative test results and test-negative animals are retested.

bulls are truly infected. When testing a herd with known *Tritrichomonas*-positive bulls, the veterinarian will likely estimate the probability of infection in other bulls in the herd to be very high, which decreases one's confidence in negative test results even further. When testing in a herd situation with a disease probability of 75%, the NPV of a single test would be around 50%.

When testing in parallel, two or more tests are applied to the entire population, or to the initial test-negative population. Parallel interpretation of multiple diagnostic tests is used when the veterinarian is concerned about making false-negative mistakes, so that a positive result in any of the tests results in a positive conclusion. The net result of testing in parallel is a gain in diagnostic sensitivity and a net loss in diagnostic specificity. Therefore the PPV is reduced but NPV is improved. Testing and interpreting in parallel is commonly done if tests are fairly easy and inexpensive, or if it is more important to identify all animals with the condition than to avoid false positives (Figure 43.3).

Summary

Interpretation of diagnostic tests is an important component of clinical veterinary practice. Veterinarians use a variety of testing methods including physical examination, laboratory testing, diagnostic imaging, and performance record evaluation. The diagnostic endpoint may be a physical diagnosis of pregnancy, attainment of puberty, or adequate quality and quantity of sperm; or a medical diagnosis of reproductive tract pathology, presence of an infectious pathogen, or abnormal hormonal status. Proper interpretation of test results requires an understanding of how diagnostic sensitivity and diagnostic specificity as measures of test accuracy and prevalence of the condition affect the interpretation of an individual result. For many diagnostic questions, the proper use of more than one test interpreted either in series or in parallel allows veterinarians to optimize diagnostic accuracy and the economic return for the testing strategy.

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44

Breeding Season Evaluation of Beef Herds

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Introduction

Cow-calf operations that optimize their cost of production and production efficiency should remain competitive through the next cattle cycle [1–3]. There are multiple factors that affect production efficiency of the cow-calf enterprise, but the reproductive performance of the breeding herd plays a key role in sustained profitability. Measures such as pregnancy rate, calving percentage, calving distribution, and percentage calf crop all affect pounds weaned per exposed female. Reproductive and calf performance measures are highly correlated as the level of reproduction of the breeding herd has a direct impact on the number of calves weaned, their age at weaning, and their weaning weight. It is critical that disease and management factors having a negative impact on reproductive efficiency are identified and corrected to ensure optimum productivity of the operation.

Over the years, the process of evaluating reproductive performance in beef herds by veterinarians has been well described [2–4]. Whether referred to as a breeding season evaluation, reproductive profiling, or "pregnancy analytics," this process requires that the producer provide or the practitioner construct adequate herd records to facilitate production evaluation and problem identification. By comparing the current level of production to historical trends on the ranch or known benchmarks of similar operations, needed changes in herd management can be identified and cost-effective solutions implemented. Once this process is in place, annual evaluations become easier to perform and historical trends are identified to document progress and prevent future losses.

Data Collection

The usefulness of available herd information is totally dependent on its accuracy and its relationship to meaningful reproductive performance measures [2, 4]. Data not meeting these criteria are not useful and only make the analysis more difficult for the practitioner to perform. Surveys indicate that as herd size becomes larger, producers are more apt to utilize individual identification in cows, keep production records, and capture that data electronically [5]. However, many smaller operations are capturing data using well-organized hand-written templates. If records are incomplete or inaccurate, it may be more productive for the practitioner to help design a system to fit the needs of the operation. Regardless of the system used to capture data, it is only useful if records can be analyzed and used to make management decisions.

The concept of "event management" has been described as a means of collecting production data, programming herd health services, and providing opportunities for client education [6]. There are major events that occur on an annual basis in the management scheme of any cow/calf operation. These events are repetitive and cyclical, and will follow each other in succession. Examples of major events include calving, branding, and grass turnout, the breeding season, summer calf management, fall weaning, and pregnancy examination. From the standpoint of the client and practitioner, these events represent extensive investments in labor, commodities, and services that are needed to manage the herd at these particular points in time. It is also an opportunity to establish a system to capture important production parameters and inventory numbers to be used for

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evaluation of herd performance. These numbers may be collected as animals come through the chute, written on clinic invoices, or recorded by the producer and provided to the practitioner for evaluation. This is where the breeding season analysis starts – capturing inventory and production numbers at particular points in time that are driven by herd management procedures.

The production numbers required to begin any analysis have been discussed in depth in other publications [2, 3, 7]. It is important to begin with "the end in mind" before data are collected to ensure that important parameters are tracked so the analysis can be completed. Many of the indices used to evaluate performance are ratios, so these numbers need to be identified and prioritized in any data collection system. As groups of breeding females are processed, their identification number, age, breed, body condition score, pasture location, and estimated days pregnant should be recorded. Additional numbers such as frame score and body weight can be utilized provided they are captured at the speed of processing and will be used to evaluate reproductive performance. Reproductive performance of individual breeding groups within the operation should be evaluated and compared as well. This allows the practitioner to better understand where reproductive shortfalls are occurring based on factors such as breeding group, cow age, or a particular pasture.

The bull battery represents the other half of the reproductive equation. Bull ID number, pasture assignment, length of the breeding season, and prebreeding soundness examination results should be available to the practitioner in the event of decreased reproductive performance [2, 4, 8]. Bull selection is critical if producers are to match the genetic base of the cow herd with the forage base on the ranch. Ensuring bulls are selected for needed calving ease, growth, and carcass characteristics will optimize calf survival and value. Bulls must also be evaluated for structural correctness and need to monitored to ensure that they are servicing females during the breeding season. The ability of the bull battery to get females pregnant early in a limited breeding should never be overlooked as a means of increasing herd reproductive performance.

Weaned calves are the primary income source for cow-calf operations, while weaning percentage, feed costs, and depreciation costs are important indicators of profitability [1, 2, 4]. Larger operations that market heavier calves tend to be more profitable as well. The length of the calving season and calving distribution will have a huge impact on weaning weights due to their effect on calf age at weaning [2, 4, 9]. Calves tend to gain at a consistent rate during the suckling period, so it is not surprising that calves born earlier in the calving season will be older and heavier at weaning. The weaning weight difference between early- and late-born calves from the same herd has been shown to favor the older calves by as much as 80–120 lb [2, 9]. It has also been shown that early calving heifers had significantly improved productive longevity compared to their later calving herdmates [10]. Producers commonly record the individual birth dates of calves, so this may serve as a starting point for program analysis. It is critical that the practitioner help clients understand this relationship between reproductive performance, calf weaning weight, and female longevity when evaluating production shortfalls.

The nutritional management of the cow-calf operation is critical to reproductive success of the breeding herd [2–4]. In addition, the cash cost associated with feeding the cow herd represents the largest annual expenditure for most operations. The nutritional management of the herd can be monitored using body condition scoring at critical times that match the female's production calendar [11, 12]. Body condition should be evaluated at pregnancy examination, 60 days prior to calving, at prebreeding, and before the start of any supplementation program. It should also be monitored through the grazing season to ensure breeding females are maintaining condition. Nutritional monitoring not only gives a current estimate of how the herd is being managed but also provides a baseline to identify potential nutritional shortcomings and needed feeding changes before the next production event. Body condition scores should be stratified across different management groups (age, breed, pasture location) in order to evaluate the impact of nutrition on reproduction across the entire herd. Females in poor body condition can be sorted and segregated into a single group and fed to gain needed weight. Nutritional management and monitoring are critical to maintain adequate reproductive performance and calf health, and identify the best way to allocate expensive feed resources.

Analysis of Herd Records

When evaluating the success of the breeding program, a traditional measure that is commonly used is pregnancy percentage. This measure is calculated by taking the number of animals pregnant in the individual group or herd and dividing it by the number of exposed females. While this measure is easy to calculate and has some value, it gives no additional insight into important factors such as the length of the breeding season, bull to female ratios, grazing conditions, or cow characteristics within the group. A more complete analysis of herd performance would enable the veterinarian to evaluate when individual cows became pregnant and the percentage by each estrous cycle. This type of analysis could also begin with calving dates stratified by 21-day intervals through the length of the calving season. A complete evaluation of herd reproductive and calf performance will also take into consideration

the replacement heifer program, the herd's nutritional status, and bull characteristics [2–4, 7, 8]. This type of analysis will lead to a better understanding of production shortfalls and the needed intervention strategies to correct them. It also leads to clear lines of communication to ensure that needed changes are carried out.

If accurate production records already exist, variations in female reproductive performance can be assessed by cow age, breed, frame score, and body condition score [2–4]. However, in the author's experience it is common to work with producers that realize there is a production shortfall (reproductive or calf performance) but have little in the way of useful documentation to aid the practitioner in their analysis. In these cases, it is incumbent that the veterinarian work with the producer to design a system that captures the needed information and is simple to use. Well-organized hand-written records will nearly always suffice to begin data collection.

Data collection must fit the normal working pattern on the ranch, whether those events are associated with breeding female management or calf working [6]. Two common events often provide the first opportunity to begin data collection – the beginning of the calving season or herd pregnancy examination [2, 4]. If the producer can capture birth dates, inferences can be made as to length of the previous breeding season, the pattern of conception, and expected weaning performance. The producer should also record the number of females and their calves turned out to grass for the breeding season that follows. If data are first collected at pregnancy examination, the veterinarian can evaluate the overall success of the breeding season and the pattern of conception, and utilize body condition scoring to establish a baseline for the nutritional management of the herd. The number of calves weaned and their weights (either individual or group) would be captured at this time as well. This type of record system is event and animal inventory driven and can be used as a starting point to begin benchmarking herd performance.

A set of easily defined production benchmarks is given in Table 44.1. In order to calculate these measures the producer is required to collect only nine inventory or production numbers throughout the course of the year [13]. All of these numbers are associated with major management events that take place in most cow-calf operations and oftentimes the practitioner is present to deliver a component of the health program. These measures are meant to act as a starting point for the producer and veterinarian to begin the process of herd evaluation. While these benchmarks are certainly not all inclusive, they do represent economically important reproductive and calf performance measures that can affect profitability of the ranch.

Pregnancy percentage is one of the most commonly used benchmarks to evaluate reproductive performance (Table 44.1) [3]. This measure is easily calculated but when used alone is of limited value. Pregnancy percentage does not provide any additional information in terms of when conception occurred (fetal age), the length of the breeding season, or the distribution of the pregnancies. It is also important that this measure is based on the total number of females that were exposed for breeding during the previous breeding season. Failure to account for female inventory changes during breeding or being unable to palpate all exposed females may affect the overall accuracy of this benchmark. It is much more useful when combined with calving or pregnancy distributions to better evaluate reproductive performance.

Table 44.1 Production evaluators of reproductive and calf performance.

1) Pregnancy percentage = (number of pregnant cows/exposed females) × 100
2) Calving percentage = (number of females with full-term calf/number expected to calve) × 100
3) Calf death loss percentage
   - Calving season death loss = (number of dead calves during calving season/number of females with full-term calf) × 100
   - Suckling calf death loss = (number of calves weaned/number turned out to grass) × 100
4) Weaning percentage (calf crop percentage) = (number of calves weaned/exposed females) × 100
5) Pounds weaned per exposed female = (total pounds of weaned calves/exposed females)

Source: [13].

Calving percentage is important in evaluation of the overall herd health and nutrition program. This measure is directly related to the level of fetal wastage occurring after females are determined to be pregnant. The inverse of this measure would be the late-term abortion rate. When attempting to determine the level of late-term abortions, it is important to identify cows that were called pregnant but failed to calve. With this benchmark, all full-term calves are included in the numerator even if they are stillborn. The number expected to calve is based on the pregnant female inventory present on the ranch at the beginning of the calving season. Depending upon the culling criteria of the ranch, both pregnant and open cows may be culled prior to the start of calving. Therefore exposed females are not used to calculate this benchmark.

The calf death loss benchmark is divided into two different periods (Table 44.1). This is because it is important to determine if mortality is occurring during the calving season or suckling period. Since the cause of mortality varies depending upon the age and production stage of the calf, understanding when death loss occurs is critical to the establishment and refinement of the herd health program. For death loss during calving, only full-term fetuses are counted in order to differentiate this measure from

late-term abortions. Mortality during the suckling period can be captured with two inventory measures that require counting the calves turned out to grass after calving and those calves present at weaning.

The last two benchmarks in Table 44.1 provide an evaluation of weaning performance and are highly correlated with ranch profitability [1, 2, 4]. Weaning percentage or percentage calf crop compares the number of calves weaned to the number of females exposed to produce those calves. Pounds weaned per exposed female requires that either group or individual weaning weights be recorded, totaled, and then divided by the number of exposed females. Both of these measures are affected by reproductive performance, calf mortality, bull selection, calf growth rate, animal health, and the nutritional management of the cow herd. These measures give an overall assessment of how successful the operation is in maximizing saleable pounds and income. While acceptable levels of production will vary by location and operation type, reasonable benchmarks for US mid-western herds are given in Table 44.2 [7, 13].

Once pregnancy data and calving records are collected, it is possible to construct distributions or histograms [2–4]. These figures exhibit the number of pregnancies or calvings that occur in each 21-day period of their respective seasons. Fetal aging at the time of pregnancy examination is required in order to construct the distribution. Pregnancy distributions should be built for the entire herd and then broken down into specific management groups based on breed, age, body condition score, or pasture location, as deemed necessary by the practitioner [2]. Reasonable targets for mature cows (4–8 years old) using a limited breeding season (45–75 days) include having at least 60% of the females conceive in the first 21-day period and less than 10% open (Figure 44.1) [2, 3, 7]. These targets may vary with different female groups or environmental conditions. Ideally, calving distributions would have more females in the first 21-day period since open cows would not be counted.

A prolonged breeding season often accompanies poor reproductive performance in herds without a routine herd health program (Figure 44.2) [2]. This type of "back loaded" distribution has nearly half of the females delaying conception until after 63 days of the breeding season or remaining open. This distribution may be caused by poor nutritional management, especially when found in young cows. Other factors contributing to this profile could be reproductive pathogens that are causing fetal wastage early in gestation [14]. When evaluating these herds, the practitioner should emphasize those factors that influence conception in the first 21-day period. Body condition scoring prior to breeding and at pregnancy examination will help to rule out nutritional mismanagement. If reproductive disease is suspected, combining pregnancy or calving distributions with information gleaned from the calculated calving percentage and calf mortality will aid the practitioner in determining when losses are occurring. This will give insight to the possible pathogens involved and guide the diagnostic protocol [14].

Table 44.2 Suggested production benchmarks for mid-western herds.

Pregnancy percentage	≥90
Calving percentage	≥97
Late-term gestational loss (%)	≤3
Total calf death loss (%)	≤5
Weaning percentage	≥85
Pounds produced per exposed female	≥500

Source: [13].

The prolonged breeding season depicted in Figure 44.2 will also negatively affect calf weaning performance. The end result of delayed conception and prolonged calving will be calves that weigh less at weaning due to their younger age. These calves will be very uneven in size and body weight, which will decrease their total value [2, 4]. Developing replacement heifers will also be difficult due to the wide range of weaned heifer ages and weights. By helping producers understand the relationship between reproductive performance and calf weaning weight, the practitioner is in a position to recommend cost-effective management changes that will impact ranch profitability.

The pregnancy distribution depicted in Figure 44.3 is not uncommon following bull failure in smaller operations that use single-sire mating groups [2]. The breeding season began well, but female conception ended abruptly. This is commonly the result of a bull injury or the dominant bull becomes subfertile and prevents other bulls from breeding cows. This injury may be a lameness issue but it is often associated with a penile hematoma or preputial laceration. Unfortunately, in many cases, the breeding season is lost by the time the injury is discovered and the bull replaced. Extreme environmental temperatures that cause the bull battery to decrease breeding activity and seek shade may also give this type of distribution. This effect may be amplified in those operations that utilize fungus-infected fescue pastures as the dominant forage. The combination of extreme environmental temperature and heat retention due to fescue grazing will decrease semen quality and libido of the entire bull battery. This will also be a concern when young, overconditioned bulls that have been developed in dry lots on high concentrate rations are turned out for pasture breeding.

Regardless of the form or relative complexity, the purpose of a breeding season evaluation is to define production problems and put in place management solutions. Accurate historical production records must be provided by the

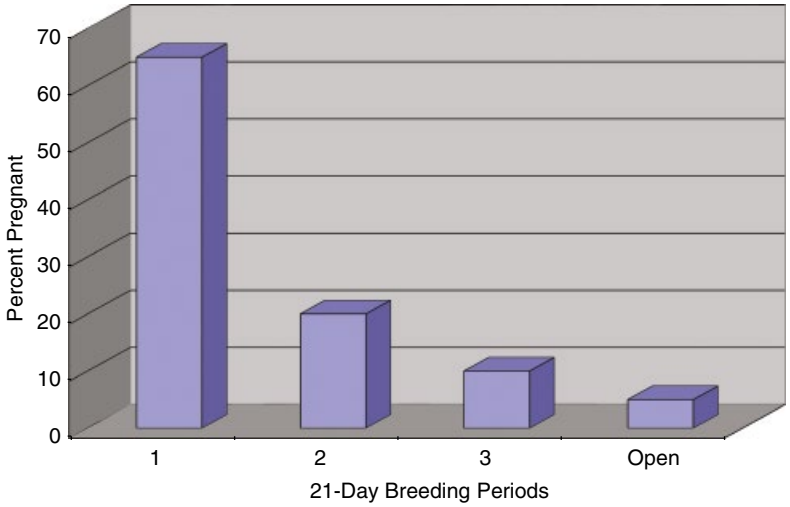


Figure 44.1 Pregnancy distribution showing percentage pregnant by 21-day periods.

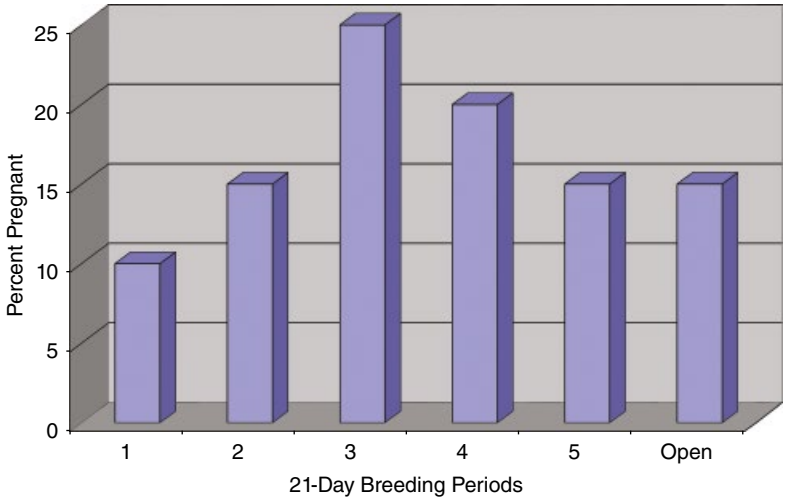


Figure 44.2 Pregnancy distribution depicting a prolonged breeding season, poor conception rate per 21-day period, and a large number of open cows.

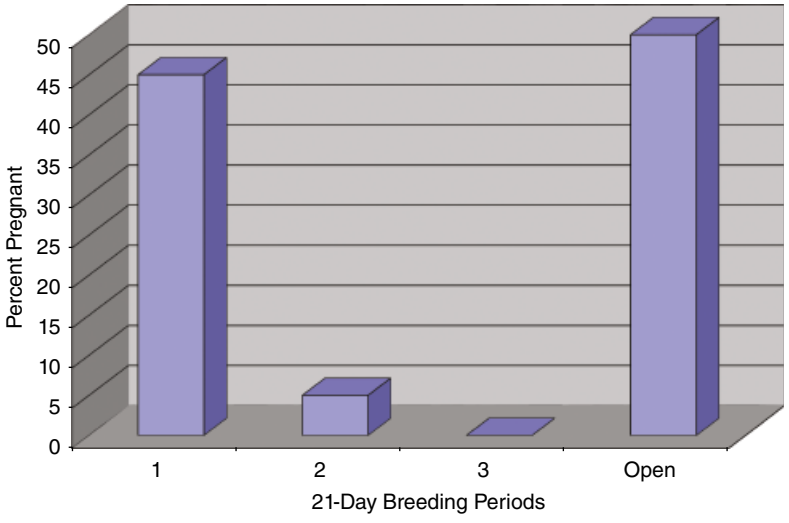


Figure 44.3 Pregnancy distribution in a spring-calving herd following multiple bull failure.

producer or designed by the practitioner to ensure the collection of clinically relevant data. Definition of the problems will include not only the magnitude of the production loss but also which management group on the ranch is affected. The etiology of the production shortfall, whether management or disease related, must be determined. Then management strategies are devised and put in place to ensure a return to optimum productivity of the breeding herd and their offspring. Monitoring of herd production is continued on an annual basis to make sure that the herd is meeting the critical benchmarks as defined by the producer and practitioner. This type of program ensures proactive monitoring of herd performance and increased profitability of the cow-calf enterprise.

Summary

Reproductive efficiency of the breeding herd is an important component that drives ranch profitability. Optimal reproductive performance is not strictly a function of the absolute number of cows that become pregnant, but also includes the timing and distribution of those pregnancies. Because weaned calves marketed represent the primary source of income for these operations, it is critical that the relationship between herd reproduction, weaning percentage, and weaning weight be emphasized to the producer. The success or failure of the breeding season and calf crop can be affected by a wide array of management and disease factors. The practitioner is often asked to provide input on problem identification, trend recognition, and cost-effective solutions. This process is made much easier if the producer can provide accurate records that are current and track clinically relevant outcomes. Once management strategies are put in place to correct a production shortfall, these records become a vehicle that allows for the annual evaluation of changes in herd productivity. This type of program will become very important to these operations, giving veterinarians a unique opportunity to provide this type of service to these clients.

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45

Dairy Herd Record Analysis

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Introduction

Reproductive efficiency is a key driver to dairy performance and profitability. Dairy managers should keep sufficient records to provide an understanding of various components of the reproductive program. This chapter will discuss the justification for records analysis, general principles for conducting an analysis, and explore various components of both the adult and heifer reproductive programs. Before analyzing a dairy's records, there should be a clear understanding of which questions the user wants answered. Specific reasons to analyze dairy reproduction records include the following: to monitor herd performance, troubleshoot performance issues, and collect research data (this last item will not be discussed in this chapter).

Records monitoring should be part of the herd veterinarian's oversight responsibility. Monitoring involves a check on both how well farm personnel are following protocols and how well the program is performing. Records can also be monitored periodically to check on a dairy's progress toward predetermined goals. *Monitoring example: Metritis incidence will be monitored monthly and an investigation to identify risk factors contributing to an increase will be started if it rises above 15%*. Goals should be specific, relevant, realistic, and should result in improved farm profitability or help to meet other business objectives. Goal setting should not be confused with benchmarking. *Goal setting example: Inseminate 60% of first service breeding eligible cows by observed estrus prior to the start of the timed artificial insemination (TAI) protocol without lowering fertility by [date].* Benchmarking involves the comparison of performance metrics to other farms or industry averages. Provided the benchmarks are relevant to the dairy, a comparison to other farms might help inform the management team when setting their goals. Use caution when doing this as some benchmarks may not be appropriate for the dairy. The farm may already be performing above its peers or industry average in an area, but additional profit could be gained by further improvement. Alternatively, the dairy might be performing far below a benchmark and improvement in this area will take a long time. Setting a lower, more realistic goal with a timeline for achievement would be more useful. The range seen in the reproductive metric averages shown in Table 45.1 highlights the importance of establishing farm-specific goals. Finally, a benchmark simply might not apply to the dairy or might be calculated differently among the comparators.

Troubleshooting involves a thorough investigation of a specific problem or performance concern and often is aided by analyzing dairy records. The prompt to investigate a problem can originate from herd monitoring or elsewhere. Knowledge of the dairy's production history, demographics, protocols, day-to-day operations, and long-term goals can help guide the analysis. *Troubleshooting example: Concern over a recent decline in conception rates lead to a review of farm records which showed the decline was coming from first service TAI breedings. TAI protocols were reviewed with the repro crew and uncovered a change in when cows received their hormone treatments which did not align with the farm's protocol*. Advisors without this knowledge should review these topics with farm management. An understanding of production history and herd inventory management can be gained from the records. A proper analysis should first confirm the issue exists, quantify its impact on overall herd performance, and then attempt to more narrowly define the problem.

Before any analysis can begin, a review of data quality is essential. This should be completed by discussing both the data entry practices of the farm staff and whether entered data are complete. Any analysis should be postponed until missing and incorrect data can be addressed. The scope of

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 45.1 Reproductive metric averages, standard deviations, and top 20th percentiles from 7842 dairy herds.

Value	Average	Std Dev	Top 20th percentile
Pregnancy rate: year average	19.5%	7.6%	26%
Conception rate: past 12 months to first service	40.0%	11.7%	50%
Conception rate: past 12 months to second and subsequent service	40.7%	12.9%	50%
Heats observed for year	48.2%	16.2%	62%

Source: Based on [1]. © DRMS.

the analysis will be determined by understanding what questions need to be answered and the quantity of the data available. There is a temptation to drill far down into a dataset in an attempt to find answers. Keep in mind the group sizes being evaluated when the data are segmented. As sample sizes get smaller, the more likely random chance can be causing the differences seen in the data. Expanding the time frame of the analysis can help maintain adequate numbers, but will add momentum (historical data influencing an outcome) to a calculation which may delay identifying a problem or miss it all together. Advisors will sometimes need to rely on data without strong statistical power, particularly when working with smaller dairies. In those situations, monitoring farm data for trends can still help inform management as long as there is an understanding that changes could be part of normal variation. Consider using other mathematical tools like medians, statistical process control, and lower confidence intervals when data are limited.

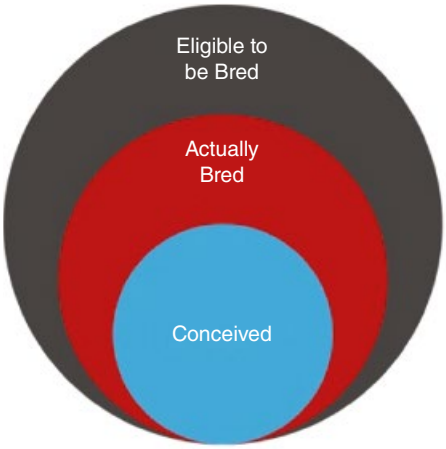
There are several farm management software programs in use on commercial dairies are suitable for data analysis. Examples of herd data tables and graphs found throughout this chapter are intended to allow the reader to become familiar with various outputs from DairyComp and PCDart [2, 3]. The values displayed are not intended to indicate recommended performance metrics. Dairy Herd Improvement Association (DHIA) standard reports can also be useful for evaluating reproductive performance on dairies, particularly the Herd Summary Report (DHI-202) and Reproductive Management Report (DHI-350) [4]. Performance metric calculations can vary from one DHIA center to another, and the advisor should review how metrics of interest are calculated by contacting the dairy's testing service.

Dairy Reproductive Matrices

The basic metrics for evaluating reproductive success on dairies are pregnancy rate (PR), conception risk (CR), also referred to as pregnancies per artificial insemination (P/AI), and heat detection rate (HDR) (Figure 45.1). PR, HDR, and CR averages and standard deviations for dairy herds included in the DairyMetrics DHIA database are shown in Table 45.1. *Example: A group of 100 cows are eligible to be bred over a 21-day period. Fifty of those cows were seen in heat and inseminated. Upon examination, 25 cows were found to be pregnant. The HDR would be 50% (50/100), the CR would be 50% (25/50), and the PR would be 25% (25/100).*

Example pregnancy, heat detection, and conception rate tables from DairyComp and PCDart are shown in Figures 45.2–45.4. These values can also be found on the DHIA Herd Summary report (DHI-202).

Although it is helpful to know how these metrics trend for a dairy, they lack enough specificity to be useful from a management standpoint. Breaking the reproductive program down into a few components will help the advisor



$$PR = \frac{\text{\# pregnancies generated over a 21-day period}}{\text{\# animals eligible for breeding over the same 21-day period}}$$

$$HDR = \frac{\text{\# animals inseminated over a 21-day period}}{\text{\# animals eligible for breeding over the same 21-day period}}$$

$$CR = \frac{\text{\# pregnancies generated form a group of inseminated animals}}{\text{\# animals inseminated}}$$

Figure 45.1 Reproductive efficiency metrics.

Date	Br Elg	Bred	Pct	Pg Elg	Preg	Pct	Aborts
7/04/16	245	129	53	241	51	21	2
7/25/16	238	129	54	237	52	22	6
8/15/16	252	156	62	247	72	29	3
9/05/16	250	156	62	242	53	22	11
9/26/16	271	185	68	259	78	30	8
10/17/16	267	165	62	260	79	30	9
11/07/16	282	177	63	278	95	34	9
11/28/16	269	174	65	265	85	32	5
12/19/16	259	173	67	251	91	36	9
1/09/17	258	162	63	251	74	29	5
1/30/17	259	164	63	255	72	28	9
2/20/17	271	153	56	266	72	27	9
3/13/17	277	179	65	268	98	37	2
4/03/17	239	155	65	234	68	29	3
4/24/17	249	119	48	246	59	24	2
5/15/17	264	135	51	249	68	27	0
6/05/17	254	135	53	0	0	0	0
6/26/17	213	125	59	0	0	0	0
Total	4150	2511	61	4049	1167	29	92

Figure 45.2 Twelve-month pregnancy rate table of heat detection rate (red) and pregnancy rate (green) in 21-day intervals and annual average at bottom (DairyComp, BREDSUM\E, Example Herd Data).

understand what is contributing to, or detracting from, the dairy's reproductive efficiency. These components are voluntary waiting period (VWP), first service estrus detection (FSED), first service timed artificial insemination (FSTAI), second and subsequent service estrus detection (SSED), second and subsequent service timed artificial insemination (SSTIA), and do not breed (DNB) policy (Figure 45.5).

A review of the farm's records should inform the advisor of both how well employees are adhering to the protocols related to each of these components (*compliance*) and how each component is working (*efficacy*). It may be beneficial to break down a dairy's reproductive program into more components, but this should be limited to where management corrections would be possible. Use caution when filtering data as bias may lead to erroneous results. Setting different goals for first lactation cows and all other cows might be appropriate. Some dairies have different VWP and/or reproductive programs for first lactation cows and all others. Also, first lactation cows tend be more fertile in general compared to older cows. A separate analysis for these groups can be informative as long as group sizes are large enough for meaningful calculations.

Not all components mentioned above will apply to every dairy. For example, a herd with a 100% FSTAI program will not have FSED to evaluate, unless employees did not follow the farm protocol and actually did breed cows observed in estrus prior to their scheduled TAI. A dairy breeding entirely on observed estrus will not have FSTAI or SSTAI fertility to evaluate. The data in Table 45.1 show the average PR, conception rate for first and second + services, and average HDR from DRMS DairyMetrics.

Voluntary Waiting Period (VWP)

There are often two different VWPs on the dairy. The stated VWP (sVWP) is what management has determined to be the minimum days in milk (DIM) a cow must be before she is breeding eligible. The actual VWP (aVWP) is when cows are bred for the first time. This can be visualized by plotting the herd's first breeding events by date and DIM (Figure 45.6). The *a*VWP can be determined by estimating the DIM when approximately 5% of the cows have been inseminated for the first time [5]. If these two periods are different, first service breeding protocols need to be

Last Date of	Bred			Pregnancies			
21 Days	#Eligible	#Reported	Pct	#Eligible	#Reported	Rate	#Abort
2/27/18	228	150	66	216	67	31	10
3/20/18	215	115	53	205	54	26	1
4/10/18	209	128	61	203	55	27	3
5/1/18	220	134	61	211	59	28	7
5/22/18	230	138	60	216	62	29	4
6/12/18	221	129	58	213	55	26	3
7/3/18	201	102	51	198	36	18	2
7/24/18	210	112	53	200	21	11	2
8/14/18	224	137	61	219	41	19	1
9/4/18	248	140	56	234	38	16	2
9/25/18	274	170	62	262	59	23	2
10/16/18	271	170	63	257	64	25	3
11/6/18	276	185	67	265	80	30	7
11/27/18	267	165	62	252	83	33	4
12/18/18	251	171	68	241	76	32	2
1/8/19	243	147	60	0	0		0
1/29/19	205	159	78	0	0		0
Total	3993	2452	61	3392	850	25	53

Figure 45.3 Twelve-month pregnancy rate summary table of heat detection rate (red) and pregnancy rate (green) in 21-day intervals and annual average at bottom (Report #126, PCDart, Example Herd Data).

Bred Number	95% CI	%Conc	#Preg	#Open	Other	Abort	Total	%Tot	SPC
1	48-52	50	1424	1416	146	135	2986	46	2.0
2	39-44	41	604	856	62	57	1522	23	2.4
3	35-42	38	321	514	58	37	893	14	2.6
4	32-41	37	174	301	31	14	506	8	2.7
OTHERS	29-37	32	169	352	* 67	25	588	9	3.1
TOTALS	43-45	44	2692	3439	364	268	6495	100	2.3

Figure 45.4 Conception risk by breeding number (DairyComp, BREDSUM\B, Example Herd Data).

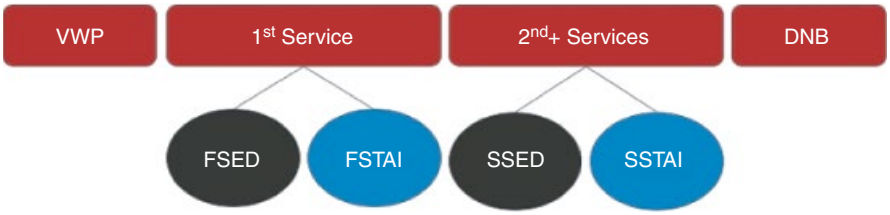


Figure 45.5 Diagram of key dairy reproduction program components.

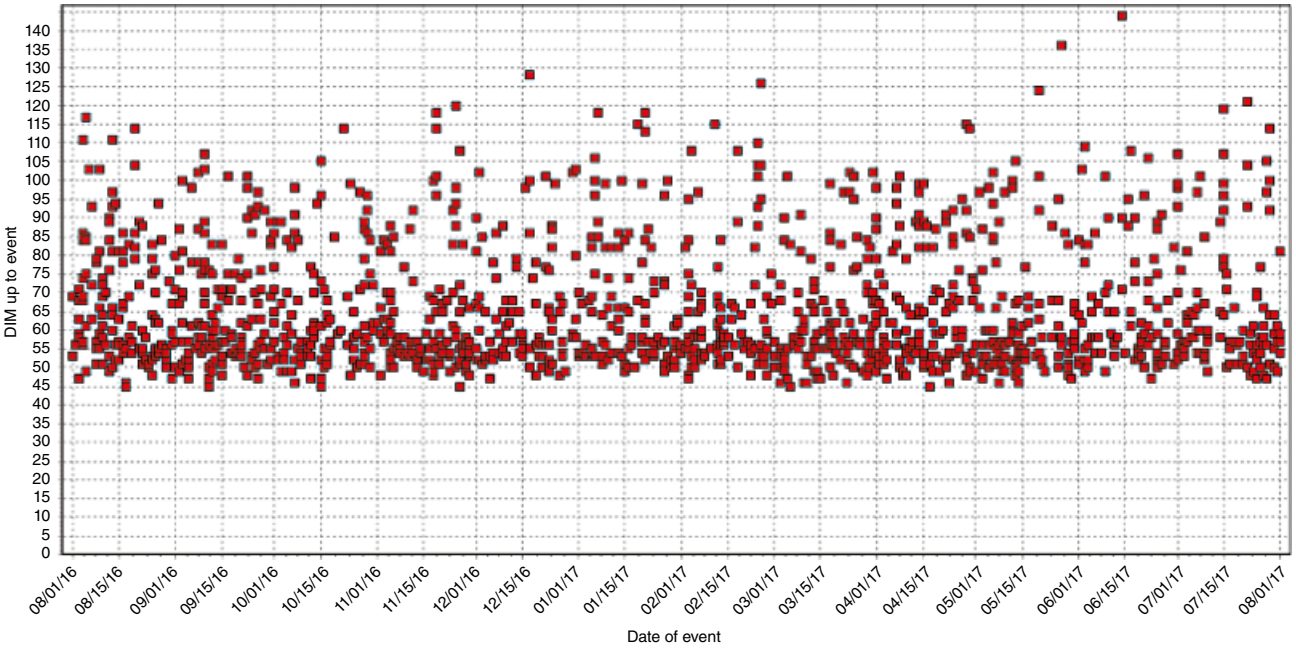


Figure 45.6 Scatterplot of first service insemination pattern (x-axis shows date of breeding event; y-axis shows DIM at first breeding) (DairyComp, Example Herd Data).

reviewed with the farm employees. Knowing the dairy's *a*VWP will also allow for more accurate heat detection and PR calculations.

First Service Estrus Detection (FSED) Breeding

For a dairy utilizing estrus detection to inseminate cows when they become breeding eligible, it is helpful to know both the proportion of eligible heats that are detected and which of those inseminations resulted in a pregnancy. Herds managing this program well will breed 50–80% of cows prior to FSTAI with conception rates ranging from 30 to 45% (Table 45.2). First service breeding success can be evaluated in the records like the example herd shown in Figure 45.7.

First Service Timed Artificial Insemination (FSTAI)

Regardless of the TAI protocol used by the dairy, checking that the protocol steps are occurring on the correct days can reveal deviations (Figure 45.8). Fertility to FSTAI pro-

Breeding Trigger | Change View | About

Trigger	%	# Bred	# Preg	# Open	Conc %	SPC	Others
No Trigger	0	0	0	0	-	-	0
M Mounting	2	29	7	22	24	4.1	45
P Pedometer	69	830	374	456	45	2.2	16
S Standing	6	75	44	31	59	1.7	3
T Time AI	22	264	123	141	47	2.1	45
TOTAL	100	1198	548	650	46	2.2	109

Figure 45.7 Conception by breeding trigger limited to first service. Highlighted in red are the two triggers primarily used by the dairy (PCDart Conception Tracker, Example Herd Data).

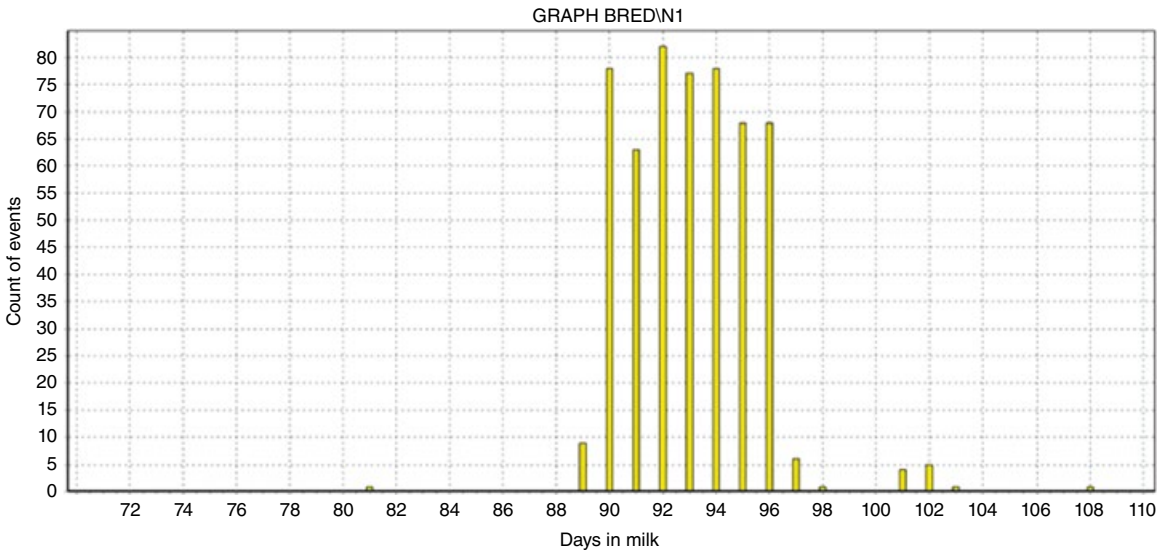


Figure 45.8 Histogram of days in milk at first TAI breeding (DairyComp, Example Herd Data).

Table 45.2 Estrus detection (ED)-based insemination rates and P/AI for first service and second and subsequent service in research settings evaluating protocols that included both ED-based AI and TAI. Refer to referenced trials for specific protocol information.

Service number (references)	Proportion of AI to ED (%)	P/AI (%)
First [6–12]	27–79	30–41
Second and subsequent [12–15]	9–78	29–43

tocols can range from 24 to 60% P/AI (Table 45.3). Consider the differences in trial designs when comparing their results to a commercial dairy's data. Were the protocols started at the same DIM or days after the last breeding? Were the number of hormone treatments and timing between treatments the same? Were cows bred if seen in heat and excluded from the TAI protocols or not? Were pregnancy examinations done around the same number of days after breeding?

Herds doing well breeding cows on first service based on observed estrus tend to have slightly lower P/AI for the cows making it to TAI when compared to herds managing first service with 100% TAI. This is partly due to a lower proportion of fertile cows inseminated by TAI and the availability of TAI protocols that maximize fertility when estrus detection is not a priority.

Second and Subsequent Service Estrus Detection (SSED) Breeding

Dairy herds willing to invest time and resources to breeding cows seen in heat after first insemination can breed between 65 and 75% of eligible cows and achieve P/AI in the range of 30–41% (Table 45.2). The wide range in heat detection efficiency displayed reflects the many variables that can influence estrus behavior including the interval between the previous breeding and the initiation of the next TAI protocol.

Table 45.3 FSTAI conception risk results in research settings. Refer to referenced trials for specific protocol information.

TAI program category (references)	P/AI (%)
GPG[a] [9, 12, 16–25]	24–45
PPGPG[b] [7, 17, 18, 22, 26–28]	31–59
GGPG[c] [12, 23, 24]	40–48
PGGPG[d] [20, 29]	33–50
GPGGPG[e] [19, 22, 28–31]	36–55
TAI+2nd PG[f] [19, 23, 26, 30, 32, 33]	36–57
TAI+P4[g] [9, 24]	25–38

[a] GPG includes variations of OvSynch protocols.
[b] PPGPG includes variations of PreSynch OvSynch.
[c] GGPG includes variations of preGnRH OvSynch protocols.
[d] PGGPG includes G-6-G protocols.
[e] GPGGPG includes variations of Double OvSynch.
[f] TAI+2nd PG includes various TAI protocols with an additional PGF2a treatment prior to TAI.
[g] TAI + P4 includes various TAI protocols with progesterone supplementation included.

Table 45.4 SSTAI conception risk results in research settings. Refer to referenced trials for specific protocol information.

TAI program category (references)	P/AI (%)
GPG[a] [15, 17, 23, 34–39]	25–33
GGPG[b] [12, 23, 37, 39]	22–40
PPG[c] [15, 36]	32–33
GPGGPG[d] [28, 35]	35–43
TAI+2nd PG[e] [15, 23, 36, 38, 40]	31–42
TAI+P4[f] [15, 36, 37, 39, 40]	31–39

[a] GPG includes variations of OvSynch protocols.
[b] GGPG includes variations of preGnRH OvSynch protocols.
[c] PPG is a short 2PGF2a and GnRH Resynch protocol.
[d] GPGGPG includes variations of Double OvSynch.
[e] TAI+2nd PG includes various TAI protocols with an additional PGF2a treatment prior to TAI.
[f] TAI+P4 includes various TAI protocols with progesterone supplementation included.

A check on the distribution of days between breedings should show a concentration of breeding activity around the typical estrus cycle length (Figure 45.9).

Second and Subsequent TAI (SSTAI)

The farm's management preference will dictate which, if any, TAI protocols are used for cows found to be open after their previous breeding. The example herd shown in Figure 45.9 enrolls cows for resynch to occur at either 42 or 49 DSLB. Research trials have demonstrated that resynchronization protocols can achieve from 22 to 43% P/AI (Table 45.4) and can be evaluated in the herd records (Figure 45.10). The proportion of cows bred to a resynch protocol will range from a low 20% all the way up to 100% for farms not utilizing estrus detection at all.

Do Not Breed (DNB) and Culling

There comes a time when a milking cow is no longer profitable to keep in the herd. Every dairy should set and review herd removal policies to maximize profitability. Herd records can be monitored for compliance to the DNB and culling policies.

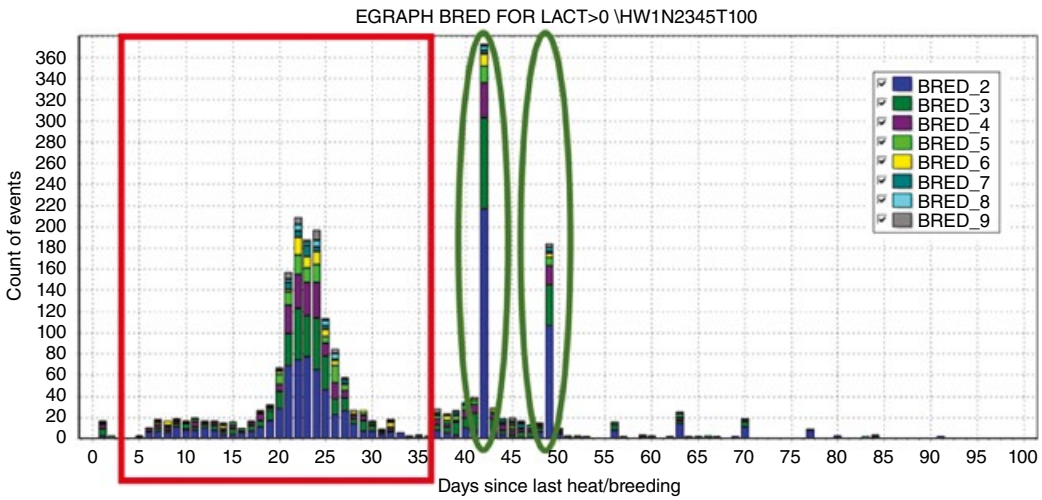


Figure 45.9 Days since last breeding (DSLB) histogram. Area in red shows the proportion of breeding events that occurred prior to a resynch protocol, which for this herd is approximately 60%. This chart also shows the farm's resynch breedings (green ovals) occurring at 42 and 49 days since last heat (DSLH) (DairyComp, EGRAPH, Example Herd Data).

Other Reproductive Analyses

Breeding Interval

A breeding interval analysis groups conception results by a series of ranges in days between the previous breeding (Figure 45.11). The majority of breedings should occur in the 18- to 24-day range, a typical estrus cycle length. Dairies can generally keep the percentage of breedings occurring in the 4- to 17-day range to less than 10% (Table 45.5). The fertility to these breedings tends to be low and a higher percentage of breedings in this category may be an indication of inaccurate heat detection. Dairies with greater than 15% of breedings occurring greater than 48 days after the previous breeding should improve heat detection efforts and/or identify open cows and re-enroll them in a breeding protocol sooner (Table 45.5).

Technician Effect

The technician responsible for breeding cows should be identified in the records (Figure 45.12). Use caution when comparing the fertility of breedings between technicians. Breeders are often inseminating different groups of cows. *For the example shown in Figure 45.12: Technician 01 only breeds cows seen in heat by weekend staff, Technician 07 does all the synchronized AIs, and Technician 10 only breeds cows still open after three breedings, i.e. "problem cows." These technicians are breeding different cows from a fertility and heat detection accuracy standpoint*. In addition, some technicians may have bred very few cows during the analysis period.

Table 45.5 Summary of breeding interval herd data from 37 dairy herds with a median herd size of 1000 milking cows.

Measurement	Median value
Pregnancy rate (standard 50-day VWP)	24%
Breedings 4–17 days	9%
Breedings >48 days	10%

Weekly/Monthly/Seasonal Analysis

Depending on herd size, tracking CR by week, month, or season can identify significant deviations in overall fertility (Figure 45.13). Identifying a real change in overall CR is not specific, but things like semen storage or handling problems will affect all breedings. Heat stress effects on herd fertility can be seen as well.

Advanced Breeding Technologies

Some dairies utilize embryo transfer (ET), *in vitro* fertilization (IVF), and ovum pick-up (OPU) protocols. It is helpful to understand how well these strategies are working and their impact on overall herd reproductive efficiency. Farms using these technologies sparingly will not likely see a significant change in their overall reproductive efficiency. Farms breeding a large number of their animals as part of an ET program could see a decline in reproductive efficiency. For example, delaying the breeding of a group of heifers to ensure an adequate number of good embryo recipients will be available for an upcoming flush will, by definition, lengthen the age at first breeding and lower the HDR among those heifers.

95% CI	Total	1	2	3	4	5	OTHERS
ReSynch	30-36	-	33-42	26-38	22-38	19-38	17-33
AFI	33-39	-	34-43	35-47	28-42	15-31	23-36
double ovsync	41-46	40-46	-	-	-	-	-
OTHERS	28-42	-	28-53	-	-	-	-
TOTALS	36-39	41-46	35-42	31-40	28-39	18-30	24-33

Figure 45.10 Conception rate by breeding code table. Displayed as 95% confidence interval ranges for FSTAI (purple), SSED (green), and SSTAI (red) (DairyComp, BREDSUM\XBO, Example Herd Data).

Heat Interval	95% CI	%Conc	#Preg	#Open	Other	Abort	Total	%Tot	SPC
1 - 3 days	-	42	8	11	0	1	19	1	2.4
4 - 17 days	17-29	23	42	143	2	1	187	8	4.4
18 - 24 days	38-45	42	344	482	27	32	853	35	2.4
25 - 35 days	29-38	34	123	244	8	12	375	16	3.0
36 - 48 days	38-46	42	261	358	14	33	633	26	2.4
Over 48 days	32-43	38	126	210	14	11	350	14	2.7
TOTALS	36-40	38	904	1448	65	90	2417	100	2.6

Figure 45.11 Conception risk table by breeding interval (DairyComp, BREDSUM\I, Example Herd Data).

Technician	%	# Bred	# Preg	# Open	Conc %	SPC	Others
01	13	251	84	167	33	3.0	32
07	47	932	402	530	43	2.3	168
10	40	803	313	490	39	2.6	109
TOTAL	100	1986	799	1187	40	2.5	309

Figure 45.12 Conception risk by technician (PCDart, Conception Tracker, Example Herd Data).

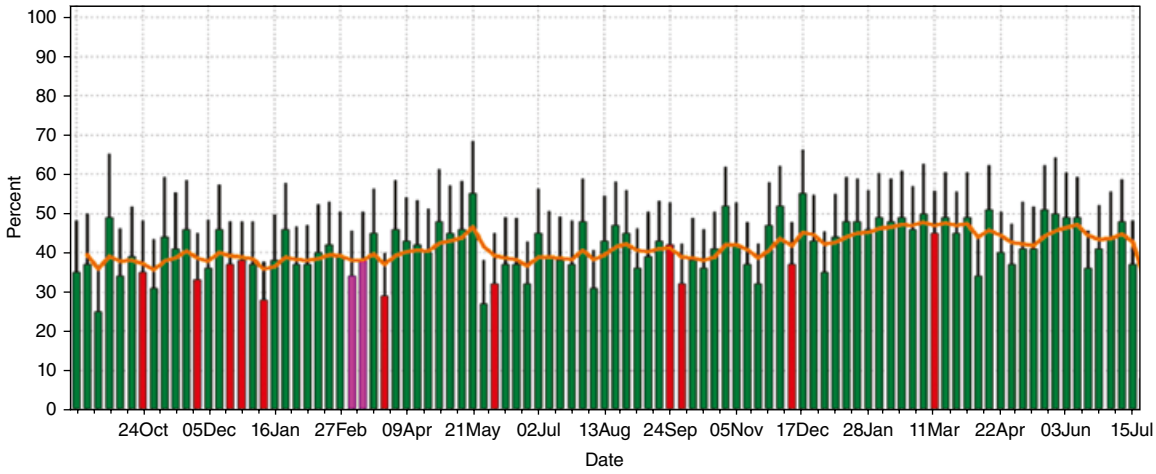


Figure 45.13 Weekly conception risk table. Red bars indicate a potential problem, light purple bars indicate more than 10% of breedings occurred within two days of a prior breeding, black lines show the upper portion of the 95% confidence interval, and the orange line is a smoothed average conception rate (DairyComp, BREDSUM\R, Example Herd Data).

Transition Cow Health

Fresh cow health conditions, particularly metritis and ketosis, can lead to poor reproductive performance. The dairy should record disease events consistently based on case definitions in order to make analysis of these data possible.

Dairy records can be used to calculate monthly disease incidence (Figure 45.14). The population at risk would be the cows that calved in the month of interest. The most recent month to be included should be one in which all the cows have completed the risk period. The example shown in Figure 45.15 shows the monthly metritis incidence over the previous year. The date range was selected to include only cows 15 DIM or more, since puerperal metritis typically occurs in the first 14 DIM and cows less than 15 DIM have not completed the risk window and could still have a metritis event. The exception to this is for animals removed from the herd prior to completing the risk period due to the disease of concern. When evaluating disease incidence, the user should select a time period that includes as many cows as possible to increase accuracy, but not over such a prolonged period of time that the calculation is no longer meaningful. If it will take several months to compile enough health event observations to allow for 95% confidence in the result, identification of a problem could be delayed, leading to additional expense to the dairy.

$$\text{Disease incidence} = \frac{\text{Number diseased in the at-risk population}}{\text{Number of the at-risk population}}$$

Figure 45.14 Calculating disease incidence.

Milk Quality/Mastitis

The dairy industry has understood for many years that cows with high somatic cell counts or clinical mastitis early in lactation are at risk of poor reproductive performance. The records review should include an analysis of the milk quality related data. Evaluating new subclinical infection risk, chronic subclinical infection, and clinical mastitis rates on a routine basis can not only help the dairy identify opportunities to improve its milk quality, but also result in reproductive improvements.

Dead on Arrival (DOA)/Calving Difficulty

Monitoring for calvings labeled "dead on arrival" (DOA) and calving difficulty can identify calving pen issues to be addressed by the veterinarian (Figure 45.16). Not only can dystocia lead

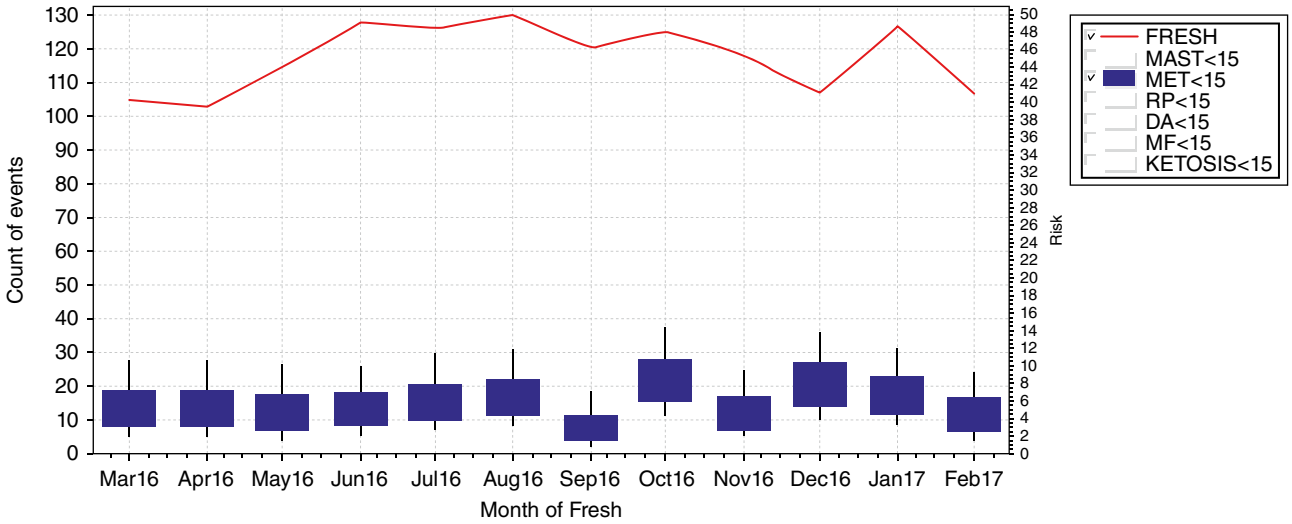


Figure 45.15 Monthly incidence of fresh cow disorders – limited to metritis events occurring prior to 15 DIM. In this example, blue boxes indicate a 68% confidence interval, vertical lines indicate a 95% confidence interval, and red line shows the monthly total of fresh events (DairyComp, EVENTS\UD15, Example Herd Data).

	TOTALS	Feb-18**	Mar-18	Apr-18	May-18	Jun-18	Jul-18	Aug-18	Sep-18	Oct-18	Nov-18	Dec-18	Jan-19	Feb-19**
ALL CALVINGS	544	26	36	39	39	65	46	59	51	42	45	51	28	17
Born Alive	525	23	37	38	37	65	45	54	48	41	41	51	28	17
Born Dead	23	3	0	2	3	0	1	6	3	1	4	0	0	0
Heifers Born Alive	302	9	16	21	22	40	29	27	28	28	21	37	13	11
Heifers Born Dead	12	1	0	0	3	0	1	4	0	1	2	0	0	0
Bulls Born Alive	223	14	21	17	15	25	16	27	20	13	20	14	15	6
Bulls Born Dead	11	2	0	2	0	0	0	2	3	0	2	0	0	0
Freemar. Born Alive	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Aborts	0	0	0	0	0	0	0	0	0	0	0	0	0	0
% Dead	4	12	0	5	8	0	2	10	6	2	9	0	0	0
% CE Score = 1	81	77	67	69	72	82	83	76	92	93	80	86	86	94
% CE Score = 4 or 5	1	0	3	0	3	0	0	3	0	0	4	0	0	0
% Female	57	38	43	52	63	62	65	52	55	69	51	73	46	65
% Twins	1	0	3	3	3	0	0	2	0	0	0	0	0	0
DCC	276	274	278	276	271	273	274	276	278	279	279	275	277	275
Days Dry	-	0	0	0	0	0	0	0	0	0	0	0	0	0
Age at Calving	705	709	725	703	705	708	719	705	713	692	708	690	682	696

Figure 45.16 Maternity pen analysis: limited to first lactation cows (PCDart Maternity Tracker, Example Herd Data).

to future breeding problems, but also it may result in fresh cow disease, poor milk production, and premature removal from the herd. Encouraging the dairy to record this information in a consistent manner will allow for routine analysis.

Lameness

Lameness can be a costly health problem for some dairies and can detract from a herd's reproductive efficiency (see Chapter 42). Dairies should keep adequate records to inform management on the incidence and causes of lameness. Records should separate new lame events from routine trims, rechecks, and chronic lameness. The herd advisor should monitor the records for lameness treatment rate (number of limb cases per hundred cows) trends, predominant lesions, DIM patterns, and lactation differences [41]. Understand that lameness is often underreported and should be cross-referenced with periodic locomotion scoring [42].

Abortion

Estimating abortion rates and setting goals for a dairy is difficult to do. The dairy industry has calculated abortion rates in several different ways. A true abortion incidence is calculated by dividing the total number of observed pregnancy losses by the total number of pregnancies created over a defined period of time (Figure 45.17). To be accurate, the time period analyzed should be chosen to ensure

$$\text{Abortion incidence} = \frac{\text{Number of abortions occurring during the defined risk window}}{\text{Number of pregnant cows past the defined risk window} + \text{Number of abortions occurring during the defined risk window}}$$

Figure 45.17 Abortion incidence calculation.

$$\text{Abortion prevalence rate} = \frac{\text{Number of pregnancies lost over a period of time}}{\text{Number of pregnancies generated over the same period of time}}$$

Figure 45.18 Crude abortion rate calculation.

all cows included will have completed the risk window. Abortion risk exists throughout gestation, making this value inherently lagged as most of the pregnancies will have been lost several months prior to the calculation. This risk window can be narrowed if the majority of pregnancies lost on the dairy occur early in gestation, which is often the case. For dairies that generate pregnancies at a relatively steady rate, calculating a crude abortion rate can inform the dairy when making decisions to investigate a potential emerging abortion problem (Figure 45.18).

Setting an abortion rate threshold is also challenging since the timing of pregnancy diagnosis and pregnancy reconfirmation will dramatically change the observed incidence of pregnancy loss. The earlier in gestation pregnancy is diagnosed, the higher the "normal" pregnancy loss percentage will be. Also, the longer the interval between initial pregnancy diagnosis and pregnancy reconfirmation, the higher the "normal" pregnancy loss percentage. Research has demonstrated an overall pregnancy loss of 25% from initial diagnosis at 28 days up to expected calving (Table 45.6) [46]. Average late embryonic (days 28–60 gestation) mortality is 12.8%, ranging from 3.2 to 42.7% [47, 48].

Table 45.6 Reported late embryonic loss.

Pregnancy diagnosis (days after insemination)	Pregnancy reconfirmation (days after insemination)	Loss (%) [reference]
30	260	3.6% [43]
28–30	40–54	16% [44]
30	80	4.2% [45]
28	98	20.2% [46]
28	84	7.2% [47]

Flawed Reproductive Metrics

Some traditional reproductive metrics should be avoided since they will not provide meaningful information to the dairy. Calving interval (CI) is defined as the time between the current calving date and the previous calving date. Average CI is inherently lagged information and does not include cows that have not calved at least twice; therefore currently open cows are excluded. Days open (DOPN) is defined as the number of days between a cow's calving date and conception date. Average DOPN excludes cows that have not yet been confirmed pregnant and therefore will not provide useful information to the dairy. Services per conception (SPC) is defined as the number of breedings a cow had before conceiving. The average SPC excludes all cows not confirmed pregnant and therefore it is not an accurate reproductive metric.

Replacement Heifer Reproduction

Understanding how well the dairy is managing the reproduction of its replacement heifers is as important as knowing its management of the reproduction of its adult herd. Heifer reproduction analysis can be broken into a few pieces: age at first breeding and calving, voluntary wait (or weight) period, HDR, PR, and CR. The dairy's records should allow for an evaluation of the heifer program's reproductive efficiency and check on compliance to the protocols being used.

Distribution of Age at First Calving

The average age at first calving is often cited as a good number for a dairy to know about its herd. This value is not sensitive enough to be a useful metric for management. Understanding the distribution in age at first calving will help identify the proportion of heifers that did not conceive in a timely manner (Figure 45.19). The economic consequences of a delay in age at first calving for replacement animals can be estimated from these data. Further analysis is needed to understand what is contributing to the delay.

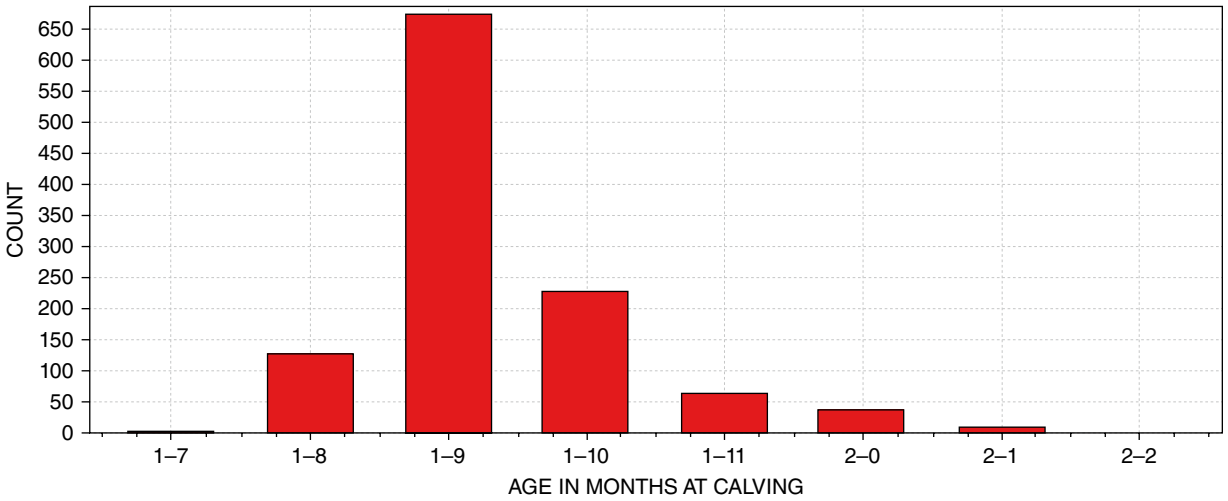


Figure 45.19 Distribution of age at first calving shown in years–months (DairyComp, GRAPH AGEFR FOR LACT = 1, Example Herd Data).

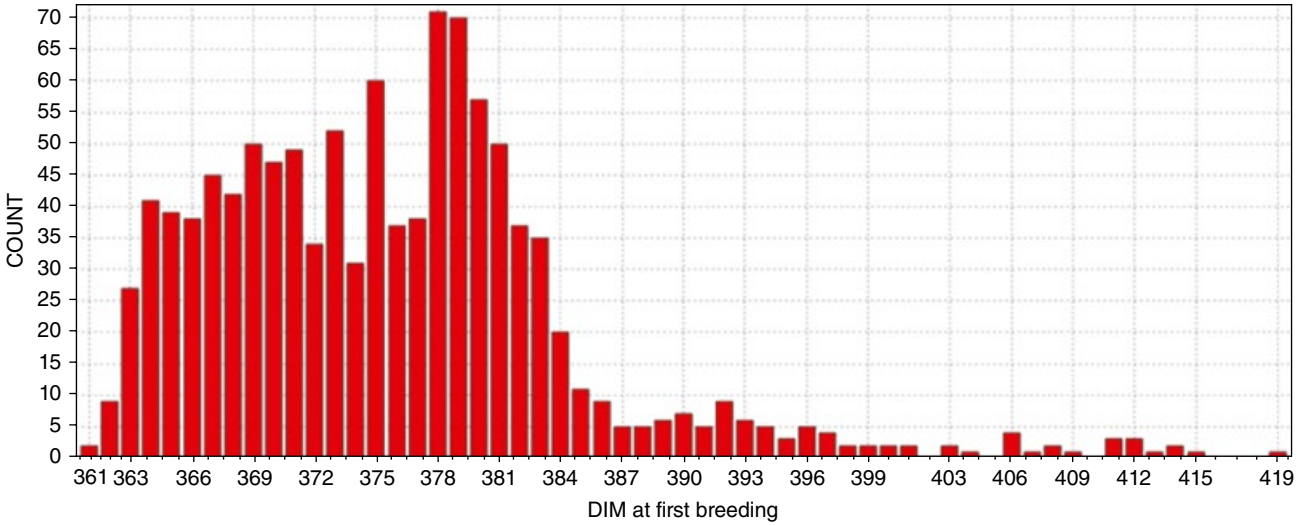


Figure 45.20 Distribution in age at first breeding shown in days of age (DairyComp, GRAPH BRED1 FOR LACT = 0, DIM is actually days of age for this graph, Example Herd Data).

Distribution of Age at First Breeding

The distribution of actual first breeding of heifers will identify the proportion of animals missing the farm's target (Figure 45.20). The delay in breeding may have been intentional or a breakdown in the breeding protocol.

Voluntary Wait Period (VWP)

Heifers should be bred when they reach sexual maturity, which is determined by body weight. When heifers reach 55–60% of their mature body weight, and they will be managed to maintain their target growth rate up to first calving, it is appropriate and financially advisable to begin breeding.

Heat Detection Rate (HDR)

Estrus detection is used by 75% of US dairies using artificial insemination to breed their heifers [49]. Heifers tend to show behavioral estrus very well, so heat detection efficiency goals will be higher in this group compared to adult herds. Well-managed dairies and heifer growers can achieve an HDR of 70–75% and have 90% of their heifers inseminated for the first time by 45 days after entering the breeding pen. Evaluating the farm's HDR based on a 365-day VWP, an age when most appropriately managed dairy heifers could be bred, can uncover intentional or unintentional delays in breeding, leading to unnecessary heifer raising costs. The example herd shown in Figure 45.21 shows the age in days when heifers were bred for the first time.

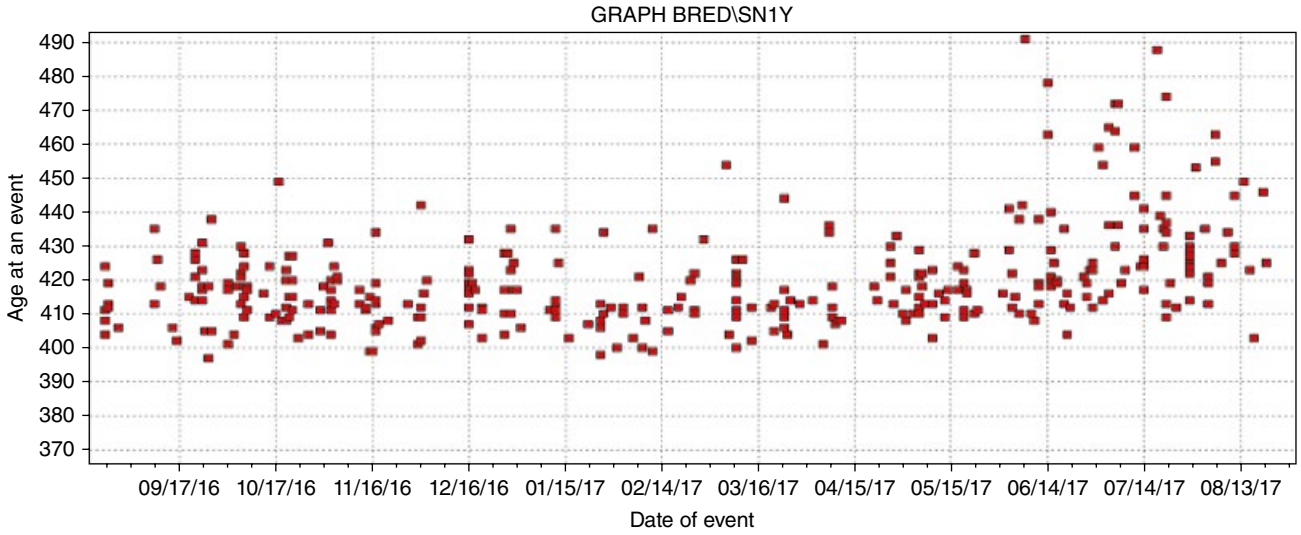


Figure 45.21 Age at first breeding scatterplot. This farm followed a 400-day VWP. Notice the breeding activity shown on the right side of the graph appearing more delayed (DairyComp, EGRAPH, Example Herd Data).

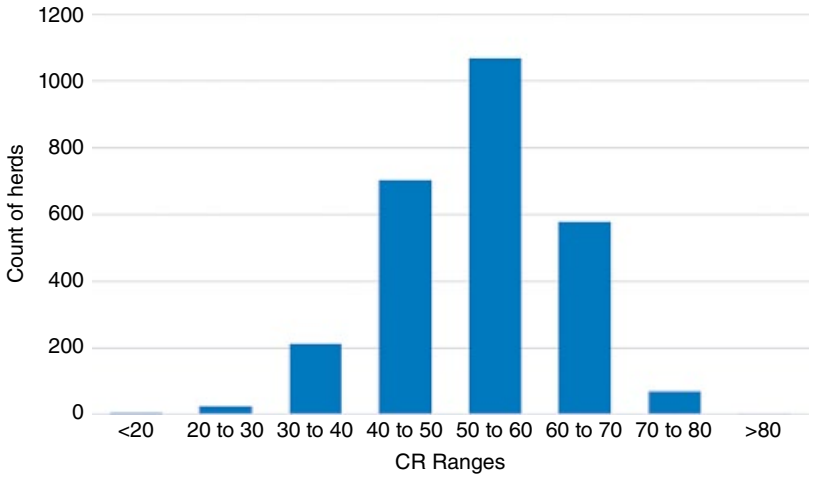


Figure 45.22 Distribution of herd mean CR for heifers bred with conventional semen using least squares mean from a linear model. *Source:* Based on [50].

Pregnancy Risk (PR)

Based on when heifers actually enter the breeding period, PR is the best measure of how effective the heifer breeding program is. The calculation is made the same as in the adult herd. Farms can consistently achieve heifer PRs of 40–50%.

Conception Risk (CR)

Dairy heifers tend to have better fertility than adult cows, so CR goals should be 60–65% when conventional semen is used (Figure 45.22). Research has demonstrated some TAI strategies for replacement heifers can be more profitable for dairies with below-average heat detection efficiency [51]. If heat detection is not feasible in the heifer herd, fixed-time artificial insemination (FTAI) protocols can achieve 50–65% P/AI [52].

Sexed Semen

Sexed semen has been available for many years and its use has been largely dedicated to replacement heifers to both accelerate the herd's genetic progress and minimize the expense by using it in the highest fertility animals on the dairy. Sexed semen technology has progressed since it was first made available commercially and fertility to these breedings is approaching that of conventional semen. Tracking fertility of sexed and conventional semen AI can be informative to management (Figure 45.23).

Semen Type	%	# Bred	# Preg	# Open	Conc %	SPC	Others
Non-Sexed	64	462	291	171	63	1.6	46
Sexed	36	265	133	132	50	2.0	15
TOTAL	100	727	424	303	58	1.7	61

Figure 45.23 Conception risk comparison – heifer breedings to non-sexed and sexed semen (PCDart Conception Tracker, Example Herd Data).

Partial Budget Template			
What is the proposed change?			
Section 1		**Section 3**	
Additional returns from proposed change	**Amount of Change**	**Additional cost of proposed change**	**Amount of Change**
Sub-total additional returns	**$0.00**	**Sub-total additional cost**	**$0.00**
Section 2		**Section 4**	
Reduced costs from proposed change	**Amount of Change**	**Reduced returns from proposed change**	**Amount of Change**
Sub-total reduced costs	**$0.00**	**Sub-total reduced returns**	**$0.00**
Summary Section			
Total Change in Benefits *(Section 1 + Section 2)*	**$0.00**	**Total Change in Costs** *(Section 3 + Section 4)*	**$0.00**
Net Change in Income *(Change in Benefits - Change in Costs)*	**$0.00**		

Figure 45.24 Microsoft Excel partial budget template. *Source:* From. [53].

Average Daily Gain (ADG)

Many dairies and custom heifer raisers are routinely weighing their replacement heifers to monitor for optimal growth. These data should be periodically analyzed to identify problems with average daily gain (ADG). Correcting barriers to growth early will minimize issues associated with getting heifers to breeding size at the farm's target age. Remember, management needs to have an accurate average mature cow body weight, which will vary from farm to farm.

Summary of Findings

At the conclusion of a records analysis, it is important to summarize and report significant findings to farm management. This should include corrective measure recommendations to address any problem areas identified. When possible, the financial implications of the problem and costs associated with proposed corrections should be included. Partial budgets similar to the example shown in Figure 45.24 can be particularly useful in this effort.

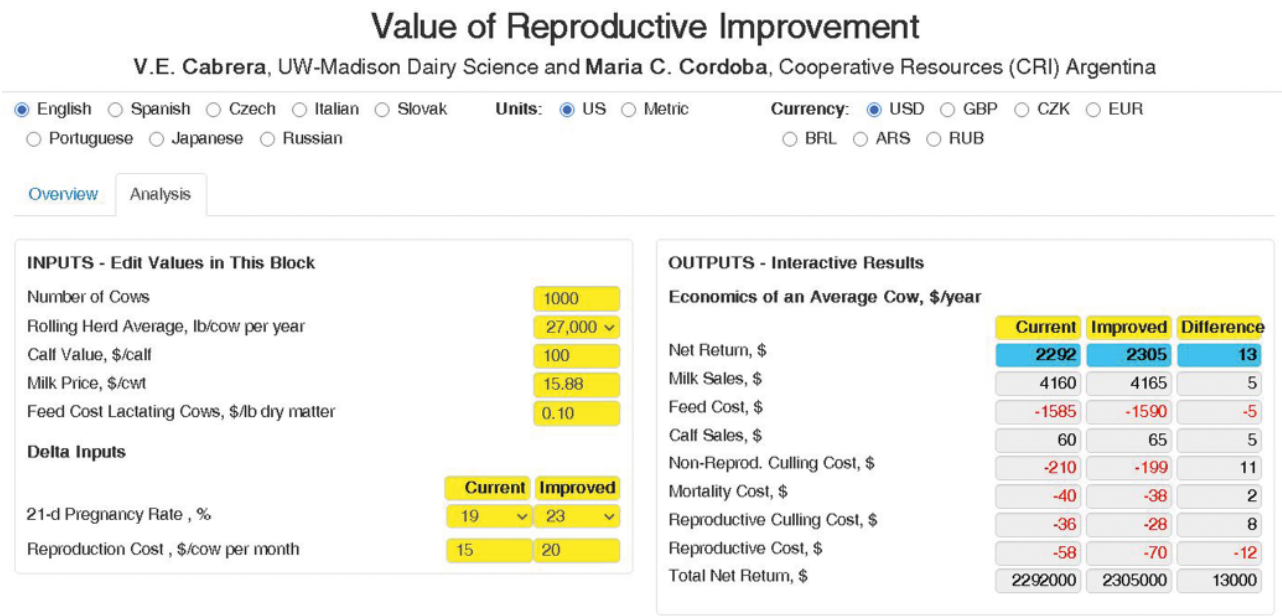


Figure 45.25 Value of reproductive improvement tool. *Source:* From [54].

The economics associated with improvements in reproductive efficiency are difficult to do without complex calculations. To assist with this, several resources are available including the Wisconsin-Cornell Dairy Repro (a reproductive programs economics analysis tool) and the Dairy Reproductive Economic Analysis Tool (Figure 45.25) [54]. With the correct inputs, these modeling tools can perform economic analyses comparing reproductive programs or estimate overall improvements.

If the analysis is part of routine monitoring, the reporting can simply be included as part of the herd health visit. An analysis conducted as part of a troubleshooting effort should be more detailed and documented. The written report can be included in the dairy's management records and shared with the farm's advisors when appropriate. A written report can be particularly useful when follow-up is needed to check on progress toward correcting problem areas, or as part of the historical record informing future investigations.

Summary

An accurate analysis of a dairy's reproductive records can be very beneficial to farm management. Problems within the breeding programs can be identified and corrected early. Goals can be tracked, motivating improvement. An advisor who can provide insight from the farm's records will be valued and play a critical role in the dairy's management team.

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46

Marketing the Bovine Reproductive Practice: Devising a Plan or Tolerating a System

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Introduction

Nothing is more common than hearing a veterinarian complain about how little income results from the practice of veterinary medicine. While such a complaint is not limited to the veterinary profession, somewhere in each of these discussions a remedy appears as if it were as simple as turning on a light or repairing a dystocia: marketing is the remedy. When it surfaces as a cure to veterinary poverty there are as many interpretations of what marketing is or does as there are individuals engaged in the discussion.

The goal in these marketing discussions is always profit, when in fact the term "marketing" implies aspects of a business many of which do not necessarily have a direct effect on profitability. A definition of marketing as adopted in 2007 by the American Marketing Association is as follows: Marketing is the activity, set of institutions, and processes for creating, communicating, delivering, and exchanging offerings that have value for customers, clients, partners, and society at large [1]. Perhaps another definition would be that marketing involves obtaining the client, satisfying the client, and retaining the client.

Principles in Marketing

Pursuit of literature about marketing frequently exposes one to the four Ps of marketing:

1) Product
2) Price
3) Promotion
4) Place.

In the context of a bovine reproductive practice, the product to be marketed would be either a service offered or some specific item such as a drug, a feed ingredient, or an instrument. The price is a fee that represents a reasonable profit and would be competitive with other businesses offering the same or similar services or items. Promotion would be those efforts taken to let prospective clients know that the practice is actively pursuing delivering what is for sale. Finally, place would be how the service or item is distributed so that clients can easily obtain the service or item at a time and location convenient to the client.

For this discussion, the assumption will be that the bovine reproductive practice would be offering a service rather than an item, and as such three more Ps come into play:

1) People
2) Physical evidence
3) Process.

There is an intangibility of service. A bull having undergone a bull breeding soundness examination (BBSE) looks much the same after the examination as he did before. A person buying a pickup, however, has a tangible object that was not there before its purchase. Clients therefore make judgments about the service by looking for clues about quality of the service as it is accomplished. This is done by assessing the skill, appearance, and efficiency of all of the people involved in the examination – from the office employees, to the assistants and the veterinarian. The physical evidence of the examination would be the professional quality of any documents such as a certificate, billing charges, and information materials or handouts that are presented after the service is accomplished. The process involves the entirety of the examination from reception of the client, the handling of the bull, the efficiency, availability, and convenience of instruments or supplies, and the adequate presentation of the results resulting from the examination.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

A Marketing System or a Marketing Plan

Regardless of the efforts, conscious or not, of any active business, a marketing system exists. Services are delivered, processes and people are in place, and promotion, pricing, and distribution of those services occur whether this system is efficient, reasonable, profitable, or successful. If one attempts to modify the system, efforts toward improvement in one aspect of the business may be detrimental to other aspects [2]. The veterinarian may be quite successful, for instance, in satisfying a client by doing excellent work in a limited number of services, but the efforts do not result in obtaining more clients. If that veterinarian embarks on a strategy of promotion that restricts the dedication to performing the service, there will be no resulting improvement in the marketing system. Likewise, if the veterinarian channels efforts into obtaining and satisfying the client but because of other competitor's superior promotion efforts the veterinarian is unable to retain existing clients, then no progress is made.

A marketing plan should therefore try to address all aspects of the system instead of merely propping up deficiencies that already exist. The resulting balance should result in a more successful, profitable, and enjoyable practice.

Three Pillars of the Marketing Plan

Before one embarks on a plan, three prerequisites must be firmly in place with commitment dedicated to each.

1) *Competence*. It is one thing to desire to operate a practice dedicated to bovine reproduction and quite another to have available the expertise needed to make it successful. Competence cannot be acquired and then left unattended. Dedication to constant study, attendance at quality seminars and conventions, interaction with knowledgeable colleagues, and familiarity and openness to new ideas and techniques are essential for a successful practice and career. This dedication and effort is expensive in both a monetary and temporal significance.
2) *Dedication of resources*. While no program will succeed if adequate funding or resources are not dedicated to it, the amount of resources requires careful consideration. The veterinarian's time is an available resource, although calculating the value of that time is difficult but necessary. Regardless, if a well-balanced and carefully considered plan is in place, the appropriate numbers should be budgeted and documented and, like all budgets, adhered to or altered as necessary.
3) *Comfort with, or at least toleration of, the plan*. Many times marketing plans and schemes will fail because there is not complete acceptance of the proposition. The competence to perform may be in place and adequate financing is available, but there is revolt at the features of the marketing plan simply because they do not lie within the comfort zone of the personality of the veterinarian. Advertising may seem too gauche, speaking to producer groups may be terrifying, and promoting oneself may strike one as arrogance. Toleration of discomfort in the pursuit of a beneficial outcome is the price one pays for personal growth. Abandoning a program because the personal discomfort overmatches any possible benefit dooms the best plans and propositions.

Devising the Marketing Plan

For purposes of discussion, the assumption will be made that a budgetary consideration of 1% of anticipated annual revenues will be dedicated to the marketing plan. This would not be enough in an entity that depends heavily on product sales (such as automobiles or hamburgers), but the 1% figure does fit well within budgets of large companies that provide services and products such as a rural electric cooperative or a pipeline engineering concern. Therefore, if the annual revenue of the practice was $500 000 per year, then $5000 would be budgeted toward the marketing plan. In this budget it is necessary to place a value on the time the veterinarian or other individual spends on the project.

Obtaining the Client

The best, but perhaps not the easiest to attain, promotion one can do is to benefit from positive word of mouth. When first embarking on a practice or when trying to urge the practice on to greater levels of revenue, the word of mouth technique may not be sufficient to satisfy the aggressive practitioner. While there may be overlap between the following categories, four divisions will be addressed.

1) *Expanding the service repertoire*. There may be requests from current or possible clients that desire a specific service, such as fetal sexing by ultrasound or embryo transfer, which is not currently available at the practice. An investment in instruments and techniques to satisfy the requests not only makes possible client satisfaction but also brings in more clients looking for the same service.
2) *Promotion of existing services*. Many times clients are unaware that certain services already exist in the practice. Brochures should be prominently displayed explaining the services, details of the services readable on invoices or receipts, and the physical evidence of these services displayed in a place of congregation

such as the reception room or laboratory. Examples of physical evidence might be ultrasound images or BBSE certificates.

3) *Development of associations that lead to programs given by the veterinarian*. Extension agencies, drug and pharmaceutical companies, breed associations, and livestock organizations are always in need of presentations. Volunteering for these types of programs offers an ideal opportunity to display one's expertise. Further, writing articles in trade magazines is an excellent method of displaying one's proficiency and knowledge.
4) *Advertisements*. Although once controversial, other medical professions have wholeheartedly embraced advertising on billboards, magazines, clothing, and other items that are given away. This is an expensive proposition and care should be taken to ensure maximum exposure to potential clients while still operating within budget and ethical restraints. A bovine reproductive practice may need to expand beyond normal practice boundaries and so advertisements in regional livestock publications or breed association magazines that the veterinarian could still service may be appropriate.

Satisfying the Client

Client satisfaction relies on much more than veterinary competence. A wise marketing plan makes use of multiple possibilities to satisfy the client and encourage the client to return for further services.

Process

One must beautify the process. The appearance of the practice, including the appearance and demeanor of the people employed, will give the client an important clue to the satisfaction possible in the service about to be received. An investment in training of the staff to this end is important, but many times it is the veterinarian who falls short in appearance and demeanor. A holistic approach embraced by all will keep everyone, including the veterinarian, working toward an efficient and professional process in providing service that is done in an attractive and pleasant environment.

Physical Evidence

As stated before, providing a service frequently leaves nothing to behold after completion of the project. It is imperative, therefore, that eye-catching, professional, easily readable, and memorable physical evidence be provided for the satisfaction of the client. In the case of a BBSE, the Society for Theriogenology (SFT) has for several years provided to its members a triplicate paper record that adheres to the standards for the BBSE as established by the SFT. Because the form was illicitly, and in violation of the copyright, copied by non-members and even non-veterinarians not following SFT guidelines, the SFT has recently produced a computer-generated online BSE form that not only embraces the best standards on BSEs, but also provides a professional-looking document that compiles statistics on all bulls entered into the program in a national and international database.

The possibilities of in-house produced documents should be carefully considered in the bovine reproductive practice. At the end of a pregnancy examination experience, even if individual records are not kept on individual animals, a recap of the satisfaction, or lack thereof, of the pregnancy status of the herd as discussed between the veterinarian and the manager should be documented on a form expressly for that purpose and a copy kept as clinic records and one left at the ranch. Placing the forms in folders, or mailing a copy back to the ranch or to the owner, ensures that the recommendations and evaluations are studied by all concerned and keeps that veterinary clinic's name in front of the client. Professional-looking forms and handouts should be developed for all procedures offered by the practice that would benefit from such a distribution.

Place

An important aspect of client satisfaction would be how the service offered would be placed, or distributed, for the convenience of the client. Consideration of this distribution of services encompasses facilities, scheduling, and availability of the veterinarian. There are successful bovine practices that are strictly based on offering the service at a haul-in facility, others that are strictly on-farm practices, and others that fall somewhere in between. Many times it may be more convenient for the client to have the veterinarian at the farm to perform services, but on a limited number of animals a much better job of control and execution of the service can be done at a quality facility managed by the veterinarian. Construction of such a facility is expensive and cannot be justified unless the veterinarian is available at the appropriate times and not elsewhere trying to perform veterinary procedures on animals inadequately captured or unsatisfactorily restrained. If change is made between one type of philosophy and another, client education becomes paramount.

Scheduling is frequently a primary source of dissatisfaction for clients. Unfortunately, conflicts will always exist whereby the client either has to wait longer than would be convenient for placement on the schedule or is postponed longer than anticipated to receive the service scheduled in the first place. Hopefully, the bovine reproductive practice will develop into a practice more amenable to scheduling and less dependent on servicing emergencies, but a great deal of patience and client education must take place for

the satisfaction of the client and the veterinarian. Experience and a knowledgeable person at the clinic (it may be the veterinarian) should smooth out some of the rough edges that always occur when several clients need services rendered at much the same time.

Finally, distribution of services works much better when the veterinarian concentrates on fewer services and elevates the quality of the services provided. A bovine reproductive practice implies specialization, and clients tolerate delays or scheduling conflict better when the veterinarian is working hard on the business and species on which the client makes his or her living.

Price

No more difficult decision is made in the practice than how to set pricing, and price is the most sensitive issue the practice will face. With adequate and fair pricing, the practice thrives, but without it the practice stumbles or sits idle. While the client is usually more satisfied with a lower price, that client may respond negatively to the corners that were cut to deliver the service with a corresponding decrease in results.

There will be advocates for either higher or lower pricing based on the benefits either side has to gain, but to the individual practice owner the ideal price means work is cheerfully accepted but at any lower level would be refused. Achieving that is difficult. This problem therefore needs serious and thoughtful consideration but it is nonetheless possible to solve. Flexibility is sometimes beneficial in price-setting, but there are perils.

The practitioner should consider the type of client the practice intends to attract but should also realize that attracting as many clients as possible would be desirable even if the veterinarian decides later that neither the veterinarian nor the client are destined for a beneficial relationship. Some, perhaps the best, clients respond well to education. For these clients, time spent in thoughtful conversation about the benefits the bovine reproductive practice can provide at a specific price is the most productive part of the relationship. The veterinarian should encourage, plan, and efficiently execute these sessions. Other clients will not respond to education and merely want a brief explanation of what is to be done and the associated price; these clients have already decided what the benefits will be and are not anxious to hear the veterinarian's viewpoint.

Thus, there are three types of clients: those that respond to education, those that will respond to education in the future, and those that will never respond to education. One cannot predict which group will be most profitable, but one can predict which group will be most enjoyable and rewarding.

While pricing is a difficult issue, the practitioner should recognize the prospect that many clients do respond to flexibility and creativity rather than responding only to competitive pricing. Flexibility and creativity can be perilous, however, so once again the practitioner should carefully weigh a new or different price structure regardless of how elegant it may seem.

Hourly versus price per task In a dairy practice or perhaps certain beef practices it may be essential to offer services by the hour. For many this is advantageous to both the veterinarian and the client. In other practices, however, there is seasonality due to set breeding seasons that results in fluctuation between being very busy and being relatively idle. In such cases bidding services by the hour necessitates a rate that must cover those times when the clinic is idle and may result in a rate that is unattractive to the client. The hourly rate of, say, a dairy practice considers the same prospect, but the dairy practitioner may have a more level production, thereby having less idle time for which an accounting must take place. An answer might be to encourage elective work during the non-busy time, even at an hourly rate.

Guarantees Many veterinarians can successfully work by a promise of outcomes. There are ethical issues at play here, but taking on a project and being rewarded by a positive outcome (higher conception rates, higher weaning weights, etc.) can be profitable if the veterinarian is confident that the strategies and procedures implemented should produce the expected positive results, especially to a reluctant prospective client. This should be a short-term proposition as the culture the veterinarian wants to cultivate is for clients to use the practice's services because they are already confident that outcomes will be beneficial.

Consulting It is hard to envision a bovine reproductive practice that does not provide consulting in some fashion, but it is equally as difficult to envision that practice dependent strictly on consulting. It would seem that by definition of the bovine reproductive practice there would be some skill (in addition to consulting) that would be brought into play, for example surgery, palpation, embryo transfer, or BBSE. While performing these procedures there is usually time to consult in the hope of developing protocols that would improve the outcome of the procedure, or at least defining what has produced a positive outcome. Consulting at this time hopefully will be covered by the charge of the procedure, but should result in positive results in the future as well as goodwill in the present. Care must be taken, however, in consulting at no charge when no other procedure or prospect of a procedure is anticipated. Telephone consultations may fit in this category.

Dispensing Traditionally, many veterinary clinics have made a sizeable portion of their profit from dispensing of drugs, biologicals, and other products. The profit made in

these activities covered other areas of the practice that were not priced at an adequate rate (such as consulting). While a dispensing practice is entirely possible, and many times quite profitable, competition from larger corporations has complicated the issue. Some clients prefer to obtain pharmaceuticals from the veterinarian, thinking this will ensure that procedures related to product administration will be done correctly. The trend seems to be the contrary to that and toward requesting prescriptions for those items necessary and purchasing them online or from other large outlets. If one is to compete in the dispensing business, care should be taken not to let it interfere with other non-dispensing procedures and one must develop skills in inventory control, purchasing, and promotion that may not already be possessed within the practice.

Retention of the Client

Attention to client retention is more than admitting existing customers are more valuable and profitable than prospective ones, it is the realization that high-quality service and exceeding expectations should be emphasized more than obtaining profits. Those clients who are loyal and engaged are the best promoters of the bovine reproductive practice.

The bovine reproductive practice would in theory thrive on repeat business from existing customers, and therefore procedures should be instigated that would keep the practitioner and the clinic in the mind of the client without seeming excessive. Follow-up phone calls from the veterinarian or staff on outcomes from previous work is warranted. Hopefully, however, a line of communication is established during the client–veterinarian relationship that lends itself to a steady stream of information between the two parties.

Newsletters

Newsletters or other means of updating clients on available procedures, current or changing issues that apply to the client's production, and diseases or syndromes recently diagnosed are well received but difficult to produce on a consistent basis. Companies that produce newsletters can be tailored to the practice, but many times do not evoke a personal touch that resonates with the client. If there is a desire to do this and there is a corresponding willingness and capital to get it done, a newsletter can prove beneficial to client retention.

Email or Social Media

Information and personal contact can be easily distributed by email or one of the social media avenues (Facebook, LinkedIn), although not all clients readily embrace such communication. As the sophistication of these outlets improves, using such devices will be a convenient and obvious method of client correspondence and feedback.

Personal Contact

Many veterinary clinics of different or general specialties already have reminder systems in place for vaccinations or other procedures. In theory, the bovine reproductive practice would not have the volume of clients that need reminding of, for instance, rabies vaccinations that exist in a small animal practice. This leaves in place the opportunity for a personal phone call on a calculated basis for business to be lined up. Herd palpations, BBSEs, and other procedures that have been done in the past and should be accomplished in the future are excellent, although there may be preferences on the client's part of how any particular information would most effectively be received. The important part is to have a system in place that accomplishes this as a matter of procedure rather than when the spirit moves. Every service provided is an opportunity to make contact in the future.

Evaluation of the Marketing Plan

Regular evaluation of the marketing plan should be part of the overall design. One should attempt to measure both objectively and subjectively what effect the plan has on the practice. While revenue or repeat business is easily determined, other determinations such as client satisfaction and staff morale are equally important but more difficult to assess. Building a consensus about what the marketing plan should do and how to measure the success of the plan may be the most important elements in the marketing concept.

Summary

Every veterinary practice has a marketing system in place. The methods for attracting, satisfying, and retaining clients or customers are present from the moment the business begins, but whether that system is effective enough for a prosperous venture depends on several factors. Conditions that are favorable for success in the present may not be present in the future.

The veterinarian launching a bovine reproductive practice is in a more favorable situation than many counterparts in other specialties of veterinary practice. Assuming the veterinarian is competent and accessible to clients desiring bovine reproductive services, and further possesses no problems with health, personality, or other distractions, no other individual has the attributes needed to deliver those services than a well-trained veterinarian. The veterinarian is the only individual who can legally provide the pharmaceuticals, techniques, and procedures needed to accomplish some of the reproductive services. No other profession can issue the health inspection papers that are important in the transport of animals, semen, and embryos.

No one else has the training to grasp and treat the correlation between sickness, health, and reproductive performance. Finally, there is a reported shortage of individuals capable of supplying all these services.

There is, however, a marked incursion into bovine reproduction made by those outside the veterinary profession. As this results sometimes in fair or unfair competition between those with a veterinary degree and those without, many veterinarians have voiced objections. These veterinarian voice complaints about legislation unfavorable to the profession, the lack of enforcement of existing laws to protect veterinarians, criticisms of the public for failing to recognize the supremacy of veterinarians, and, finally, protests about the profession itself for not providing its members with the skills necessary to make an adequate living.

Concurrently the veterinary profession, especially the bovine practitioner, is populated with talented and creative individuals capable of providing efficient and quality medical and reproductive services under less than ideal conditions. Collectively and individually there is no reason the bovine veterinary reproductive specialist should be impeded from establishing a specialty practice of sufficient profitability if the veterinarian applies as much thought, creativity, courage, and cunning into marketing as has already been made in other parts of the business.

The success rate of businesses is fairly consistent across all types of industry and failures are not always related to poor marketing. The most common complaints about the failure to succeed in bovine veterinary practice, however, can be offset by a well-conceived marketing plan, even though its execution will require courage, creativity, and the ever-present hard work.

In his book *Lessons From a Desperado Poet*, author and large-animal veterinarian Baxter Black demonstrates methods of marketing that have successfully ushered him into the difficult world of entertainment, publishing, and performance [3]. His talents are obvious, but remarkable in this book is the relentless pursuit of creative strategies and hard work that preceded his success. While many of his methods would not be appropriate in a marketing plan for a bovine reproductive practice, his example as one who pursues all avenues in his quest for markets should be an inspiration to all veterinarians.

The profession needs to claim what is rightfully and legally mandated from the federal and state level surrounding pharmaceuticals and their use, the provision of specific practice acts, and the validity of the veterinary client–patient relationship. Notifying authorities of non-veterinarians or veterinarians not in compliance is the responsibility of each ethical veterinarian regardless of the personal discomfort involved.

It should also be noted that there are valuable contributions to be made by those outside the veterinary profession and an amicable relationship with these individuals can enhance the bovine reproductive practice.

In any business, the rewards of entering that enterprise must be commensurate with the effort needed to succeed. While there is an inclination to find challenge and enjoyment from a bovine reproductive practice, if the rewards (monetary, prestige, professional satisfaction) do not justify the sacrifices (number of hours worked, working conditions, vocational hazards) such a practice will fail. In short, in order for the bovine reproductive practice to succeed on a national scale, we must attract quality veterinarians, pay them well, provide adequate equipment and safety, and spread the work among others, enabling the veterinarian to have a life outside of the practice.

It is, therefore, imperative to have the professional skills needed for bovine reproduction and the marketing abilities to entice quality veterinarians into the field. If the marketing effort fails, and with it the ability to reward veterinarians adequately, the outlook for the bovine reproductive practice fails as well.

Helping clients provide the nation and the world with high-quality, safe, and reasonably priced food is a noble occupation. Resources and support are readily available. Rewards are within the grasp of those willing to use their talents – marketing and otherwise – and their dedication to success.

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Obstetrics and Reproductive Surgery

47

Management of Vaginal, Cervico-Vaginal, and Uterine Prolapse

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Introduction

Eversion of the vagina or both uterine horns from the vulva is commonly referred to as vaginal or uterine prolapse (from *prolabi*, to fall out), respectively. It is not unusual to encounter these cases in cattle, particularly in the developing world. Vaginal and cervical eversions usually occur before calving and uterine eversions after calving. There is no relationship between occurrence of these conditions. An excellent review [1] has been provided describing the management of these conditions from the perspective of replacing, repairing, and, if need be, amputating the uterus. Noteworthy is a detailed description on manual eversion of the uterus to correct a uterine tear, a technique that involves the use of different pharmaceuticals [2, 3]. This chapter will address this condition from three perspectives: it will describe these maladies and provide remedies with representative images, both real and created; it will provide a summary of information reported in the global literature, including practical solutions provided by practitioners; and it will address these clinical situations from productive and fertility perspectives and, most importantly, from an animal welfare perspective.

Vaginal and Cervical Eversions

Eversion of the vagina is a relatively common occurrence, particularly in cattle, compared to other species [4]. There appears to be a genetic component to its occurrence in beef cows compared to dairy cows. Among beef breeds, Hereford [5], heavier breeds such as Charolais and Limousin [6], and Shorthorns are more affected than others. It has been known that *Bos indicus* breeds are predisposed to this condition, along with cervical eversion.

Many factors have been suggested to contribute to this condition [7–10], including increased intra-abdominal pressure in late pregnancy, extreme cold weather, excess perivaginal fat, prior injury to perivaginal tissues, intake of large volumes of poorly digestible roughage, poor vaginal conformation, persistence of medial walls of the Müllerian ducts [11], and changes in hormonal secretions, particularly increased estrogen as observed in late pregnancy and in estrus. It has been suggested that higher concentrations of estrogens found during pregnancy can contribute to excessive relaxation and edema of pelvic ligaments that support the vagina. Besides the above, incompetence of the constrictor vestibule and constrictor vulvae muscles may have a role in etiology [7]. This condition is observed in embryo donor cows that are exposed to hormonal stimulation of ovaries. Imbalance in calcium and phosphorus, and hypocalcemia have been considered to be contributing factors by some researchers [12]. It should be pointed out that association with hypocalcemia was suggested merely based on response to calcium therapy rather than on clinical signs of hypocalcemia. However, as eloquently suggested [12], it can be tentatively postulated that subclinical hypocalcemia could be derogatory to the internal environment of the physiologically stressed female because of pregnancy and parturition.

The majority of vaginal and cervical eversions occur in the last few weeks of pregnancy; however, the condition may occur several months before calving. In some cows, it may occur during estrus. Vaginal eversions are classified into four grades according to severity of eversion, extent of injury, and exposure of the cervix in such eversions.

- Grade 1 eversion: a small intermittent protrusion of vaginal mucosa through the vulval lips is noticed (Figure 47.1) when the animal lies down. Often this tissue becomes dehydrated and traumatized and leads to a grade 2 eversion.

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Figure 47.1 Grade 1 eversion: vaginal eversion occurs intermittently, particularly when the animal lies down.

A grade 1 eversion is readily repaired by applying any of the retention sutures described later.

- Grade 2 eversion: vaginal mucosa protrudes through the vulval lips continuously (Figure 47.2), with the possibility of the urinary bladder getting trapped in the everted organ. If left untreated, a grade 2 quickly progresses to a grade 3 eversion.
- Grade 3 eversion: the entire vaginal mucosa and cervix protrude continuously through the vulval lips (Figure 47.3), with a trapped urinary bladder and an exposed cervical mucous plug. The cervical seal may liquefy, allowing bacterial contamination of the uterus, leading to placentitis and fetal death. Often these fetuses become emphysematous and cervical dilation is insufficient for a vaginal delivery. This condition is often referred to as a cervical eversion but more correctly should be referred to as a cervico-vaginal eversion. This distinguishes it from eversion of the cervix alone, as sometimes is observed in *B. indicus* breeds. In the latter case, only the cervix everts as a pedunculated mass through the vulva.
- Grade 4 eversion: an eversion of such a duration that the entire vaginal mucosa appears necrotic and fibrosed (Figure 47.4). Infection is so extensive that the urinary bladder may become involved in it and peritonitis is a possible result.

The objective of treatment in pregnant animals is to replace and retain the vagina and cervix within the pelvic canal and to deliver and wean a live offspring. Many management and treatment options are described for vaginal and cervical eversions [1, 4, 7, 9–11, 13–34]. These can be placed in two general categories: those aimed at permanent reduction, and others that afford a temporary solution. It should be mentioned that one approach may not fit for all grades. Severity of eversion, time to expected delivery, the veterinarian's preference, and the owner's ability or willingness to manage the patient after treatment will dictate the treatment option. The elected initial treatment option needs to be changed after assessing patient response. This is particularly relevant if tenesmus becomes an issue. If the patient is close to parturition, induction is recommended to prevent recurrence prior to parturition.



Figure 47.2 Grade 2 eversion: floor of the vagina everts and stays everted. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.



Figure 47.3 Grade 3 eversion: floor and cervix evert and they stay everted.



Figure 47.4 Grade 4 eversion: either a grade 2 or grade 3 that has become necrotic and/or fibrotic. *Source:* Reprinted from *Color Atlas of Diseases and Disorders of Cattle* by R. Blowey and A. Weaver with permission from Elsevier.

Short-Term Treatment Options

Caslick's Suture

This technique (Figures 47.5 and 47.6) should be reserved only for a grade 1 vaginal eversion with no irritation that occurs close to parturition and when tenesmus is not expected to be a concern. Unfortunately, a simple Caslick's suture in many cases is insufficient. As intra-abdominal pressure increases, tenesmus begins and the suture is ripped out or the vagina begins to evert below the sutures. Furthermore, vulval softening and stretching that occur closer to parturition contribute to this phenomenon. Caslick's suture may suffice in patients that are not pregnant and have a grade 1 eversion during estrus. This is often the case with embryo donor animals whose ovaries are frequently superstimulated with hormones for the sole purpose of increasing the number of ovulations.

Buhner Stitch

This is an effective treatment for more advanced grades and those that are chronic in nature [17, 18]. To have a successful outcome, the stab incision has to be placed deeply such that the retention line is as cranial to the vulval lips as possible (Figure 47.7). A disadvantage is that it is unforgiving,



Figure 47.5 Caslick's suture being applied to a non-pregnant cow with grade 1 vaginal eversion. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

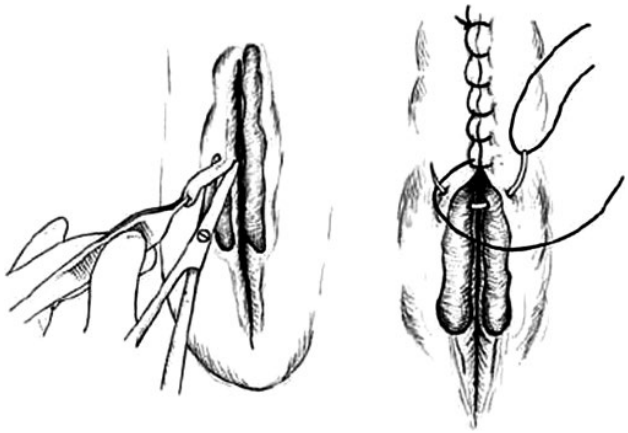


Figure 47.6 Caslick's suture procedure. *Source:* Illustration by Dr. T. Barkley.

and if assistance is not available when the patient begins to calve, severe trauma to the vulva may occur or the calf becomes undeliverable and dies in utero or the patient may die as a result of uterine rupture and fatal hemorrhage. Following epidural anesthesia, the vulva is thoroughly washed with detergent and a 1-cm stab incision is made on the midline below the vulva and as far forward as practical. A second incision is made midway between the anus and dorsal commissure of the vulva (Figure 47.7). A Buhner needle is then passed deeply from the ventral incision up one side of the vulva and out through the dorsal incision. Buhner tape (non-wicking type) is passed through the eye of the needle and the needle is retracted through the ventral incision along with the tape.

The procedure is repeated on the opposite vulval lip and the two ends are tied leaving approximatel a two-finger opening in the ventral vulva for urination. Preference is to tie a bow knot and then tie the two loops of the bow in a

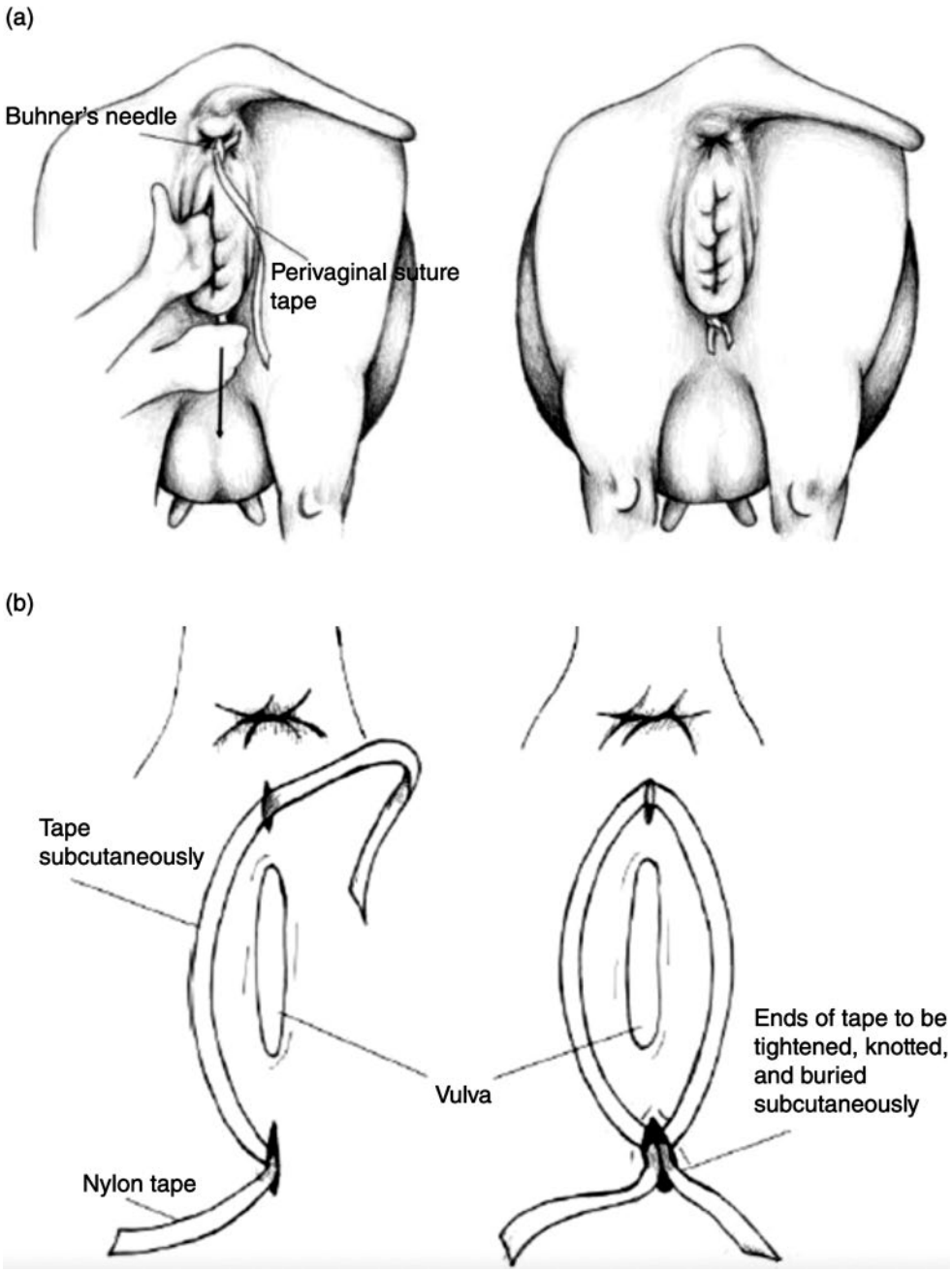


Figure 47.7 Buhner stich procedure. Note locations of dorsal and ventral retention lines. *Source:* Illustration by Dr. T. Barkley.

single square knot. This allows the knot to be loosened but to retain the suture so that if the patient should try to evert again, it can be replaced and the knot retightened. This can also be done in the evenings if observation of the patient for parturition is not possible overnight.

If the procedure is performed properly, the tape should not be visible at the dorsal incision and this helps to prevent feces tracking down the needle paths. It should be stressed to caretakers that their presence at the onset of calving is crucial for a successful outcome. If observation is a problem, medium-sized catgut can be doubled and used as a suture material; this helps in patients that are within 10 days of calving as the gut will break during parturition.

Bootlace Technique

This is not a highly preferred technique as the vulval lips are inverted and suture placement is tedious. However, it is stronger than a Caslick's suture. Following an epidural anesthetic procedure and disinfecting the vulva and

surrounding area, four to five small eyelets are made with umbilical tape or hog nose rings [11] on either side of the vulva in a dorsal to ventral line at the hair to hairless junction on the vulval lips. After placing eyelets, umbilical or Buhner tape is used to "lace up" the vulva much like a boot (Figure 47.8). As the pattern is tightened, the vulval lips invert. Again, bootlace sutures must be loosened prior to calving to avoid serious trauma to the vagina.

Horizontal Mattress (Halstead) Technique

In this technique a needle is passed through the vulval lip on the right, beginning deep at the junction of the labia with skin of the rump. The needle is continued across the vulval opening and through the left vulval lip at the same depth as the right. The needle is then passed back from left to right at the same plane as the first passage but beginning approximately 2–3 cm ventral to the first passage. This procedure can be done using a Buhner or large cutting needle. Place as many sutures as needed to close the vulva down to approximately two fingers wide to allow for urination.

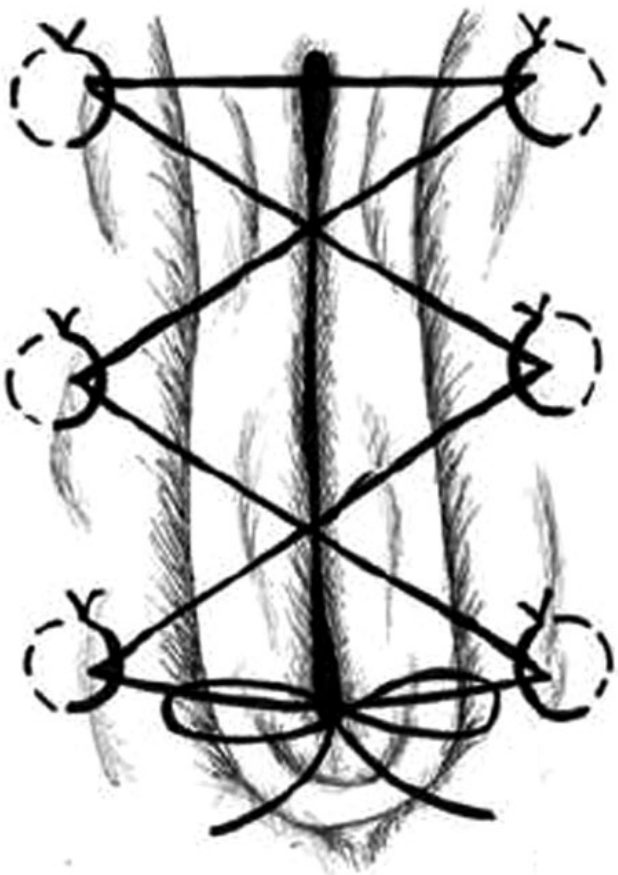


Figure 47.8 Bootlace technique. *Source:* Illustration by Dr. T. Barkley.

A disadvantage of the technique is that even if sutures are not pulled really tight, vulval edema frequently develops and can be quite severe. This can be lessened if suture tension is dispersed by "stents." Polyvinyl chloride tubing (1.3 cm diameter) can be cut to the length of the vulva and holes drilled 2–3 cm apart; similarly, wooden dowels [24] or latex tubing can be utilized. A stent is placed either side of the vulva and a horizontal mattress suture is passed through the holes and tied (Figure 47.9). This approach has good deep retention and pressure is more evenly dispersed.

Deep Vertical Mattress Technique

This is similar to the horizontal mattress pattern described above. The difference is that a vertical mattress suture pattern is used and stents are recommended to disperse tension on the vulval lips (Figure 47.10). This replacement method can also have serious consequences if assistance is not available at the onset of parturition.

Permanent or Semipermanent Treatment Options

Minchev Vaginopexy

Two Minchev techniques are described that differ only in location of needle insertion. In the original Minchev technique the needle is passed through the lesser sciatic foramen. This is an excellent prepartum method that aims to retain the vagina in the correct pelvic location by adhesion formation between the submucosa of the vagina and surrounding fascia. Anesthetic is given epidurally and the gluteal area is shaved and disinfected. In the original Minchev technique, a large S-shaped needle is threaded with 0.95-cm umbilical tape and a gauze bandage or large plastic or metal plate is attached at the tape's end; the needle is then taken in vaginally and the lesser sciatic foramen is located on the dorsolateral wall of the vagina. The needle is pushed through the dorsal area of the foramen in order to avoid the pudendal nerve. It is then pushed through the skin in the gluteal area and a suture pulled up snug (do not tie too tightly) and another button, plate, or gauze is tied into the suture. This is repeated on the other side.

Modified Minchev Vaginopexy

This modified technique is similar to the original one except that the stay sutures are placed anterior to the lesser sciatic foramen 5 cm lateral to the midline and just posterior to the shaft of ilium, providing more cranial fixation of the vaginal

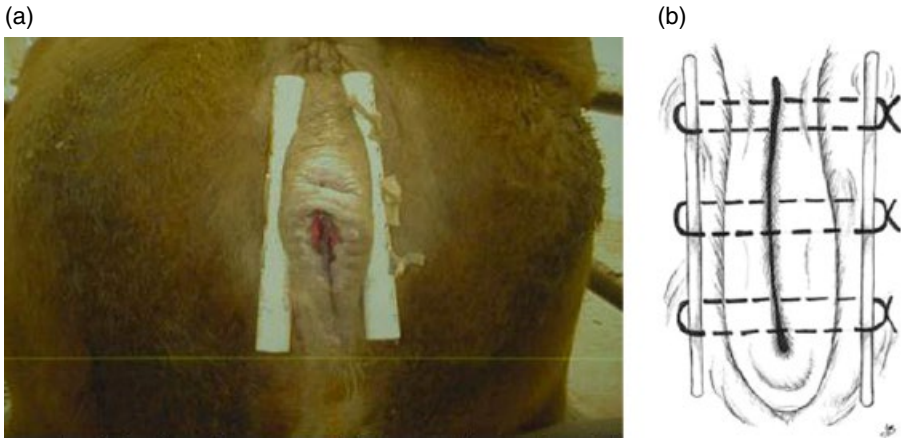


Figure 47.9 (a and b) Example of horizontal mattress technique. Note location of stents for reduction of vulval edema and for dispersion of pressure. *Source:* Illustration (b) by Dr. T. Barkley.

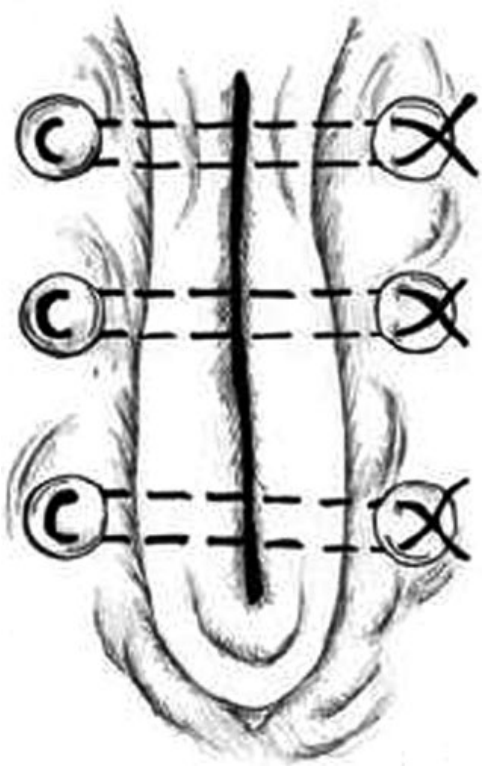


Figure 47.10 Example of deep vertical mattress technique applied where buttons are used as stents. *Source:* Illustration by Dr. T. Barkley.

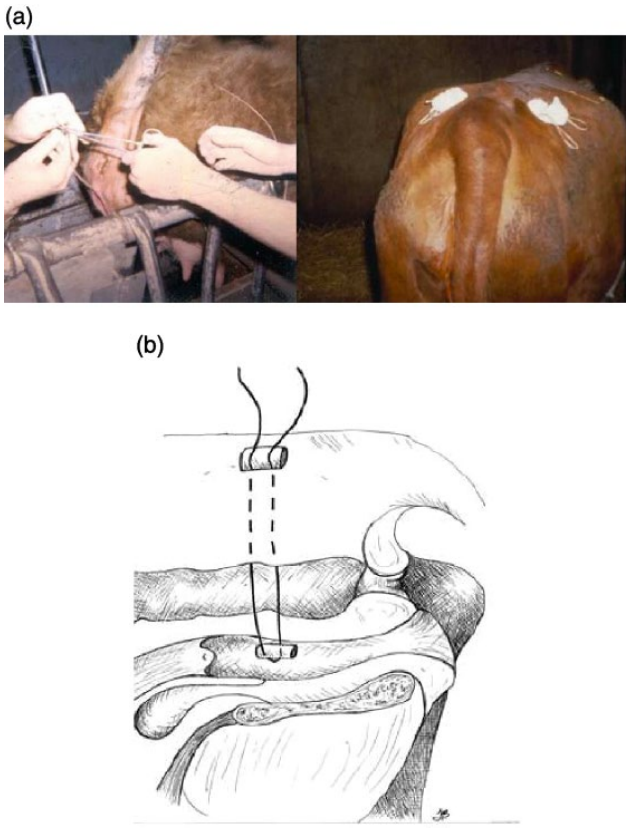


Figure 47.11 Example of modified Minchev technique (a) with schematic illustration (b). *Source:* Illustration (b) by Dr. T. Barkley.

wall (Figure 47.11). Care must be taken to avoid the sciatic nerve, pudendal artery, and rectum when placing the needle. These structures can be identified cranial and slightly dorsal to the lesser sciatic foramen. The needle is passed through the sacrosciatic ligament approximately 4–5 cm lateral to the sacrum and just posterior to the shaft of ilium.

An adaptation of this technique has been described [33] that utilizes a Gerlach needle for suture placement (Figure 47.12). The cow is prepared as above, and a Gerlach needle is introduced into the vagina with the point (tip) guarded by the thumb and forefinger. The needle is then introduced into the vaginal wall near the cervix at the 10 or 2 o'clock positions, and if necessary the vaginal wall can be retracted caudally to allow for passage through the sacrosciatic ligament to the exterior. The Gerlach needle is then



Figure 47.12 Gerlach needle (left) and Buhner needle (right). *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

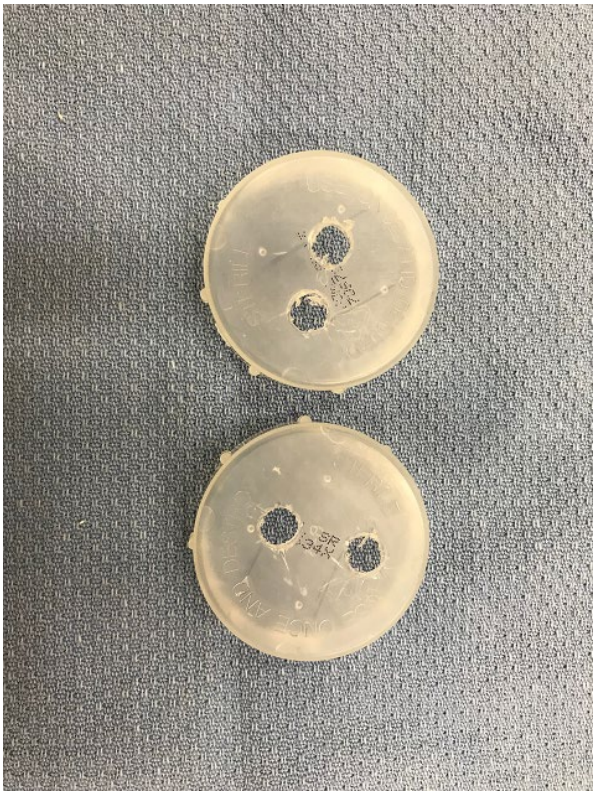


Figure 47.13 "Syringe case tops" prepared for use. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

threaded with #8 polyamide thread suture or umbilical tape and withdrawn from the vulva. A suture is cut from the needle and the two ends are threaded through the two holes previously prepared in a plastic disc that can be fashioned from 60-cc syringe case tops, sample cup lids, or the bottom of a 500-ml fluid bottle (Figure 47.13). The two tails of the suture exiting the gluteal region are then drawn taut and tied over a gauze externally (Figure 47.14) [33].

The advantages of the modified Minchev technique include the fact that cows rarely show tenesmus when the vagina is replaced and the cow can calve unassisted. However, it is recommended that sutures are removed in two to four weeks. A commercial prolapse repair kit is available (Figure 47.15; Pro-Button Prolapse, Jorgensen Laboratories, Loveland, CO). The assembled kit is soaked in disinfectant. A stainless-steel pin with a 15-cm plastic trocar is passed through the 7.5-cm plastic button and is carried vaginally. A trocar is pushed dorsal or anterior to the lesser sciatic foramen, pointing dorsolaterally to avoid the rectum, and then through the sacrosciatic ligament and biceps femoris to the skin outside. The stainless-steel pin is removed and a 6-cm plastic button is placed on the protruding trocar. Pushing down on the 6-cm button enables the 7.5-cm button to be held firmly between the vaginal wall and sacrosciatic



Figure 47.14 External view of bilateral completed modified Minchev technique; stay sutures tied over gauze externally. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

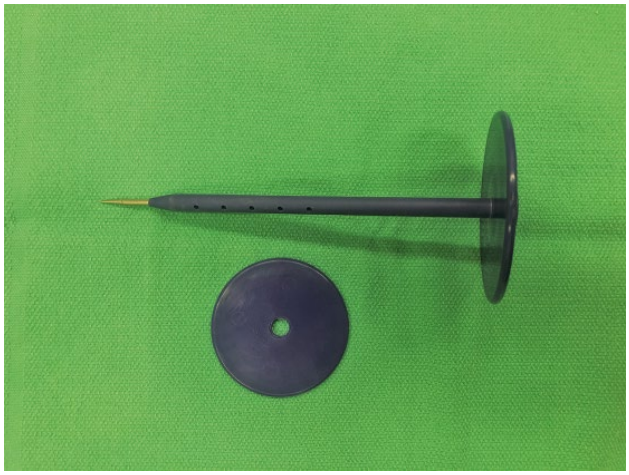


Figure 47.15 Example of trocar and plastic buttons used for modified Minchev technique.

ligament. A cotter pin is inserted through the hole in the protruding trocar. A plastic trocar is cut about 2.5 cm above the cotter pin. In severe cases, it may be necessary to place buttons on both sides of the anterior vagina. However, it should be recognized that recurrence of eversion is possible since only the dorsum of the vagina is held. Further, when internal placement is too far caudal, a partial eversion may occur, eliciting straining that results in total eversion.

Winkler Technique Cervicopexy

This technique [34] is an effective method of vaginal retention because it fixes the cervix in place, making it difficult for the vagina to prolapse. The basic procedure is aimed at suturing the cervix to the prepubic tendon. There are numerous descriptions of placing the suture in the cervix and how to suture through tendon. Approaches to suture placement are either through a left paralumbar approach [26] or through the vagina. The former approach is more time-consuming but safer, while the vaginal approach is quicker but can result in a broken needle. The vaginal approach could be done in two ways: either by including the lower half of the cervix or by just including the external os of the cervix on one side (Figure 47.16). Before placing the suture vaginally, anesthetic is given epidurally and a mild antiseptic douching of the vagina is performed. The prepubic tendon is then palpated at its attachment to the pelvic brim. As the fingers are moved laterally about 5 cm on both sides of the midline, a small triangular-shaped area free of tendon is felt. This is formed by a short band of tendon extending posterolateral to the iliopectineal eminence of the pelvis. A large curved cutting needle is bent into either a U shape (Figure 47.17, left) or other shape (Figure 47.17, right) and about 150 cm of nonabsorbable suture material is attached. The needle is then carried into the vagina and passed from the inner lumen of the external cervical ring out peripherally. The needle is grasped again and passed down in a medial to lateral direction through the prepubic tendon and carefully curved around to come up through the triangular area. The needle is grasped again, passed through the external cervical ring from periphery to center, and brought on out of the vagina. Some clinicians prefer to pass the needle through the vaginal wall initially, then pass the needle through the prepubic tendon medial to the lateral direction, and pull the needle back through toward the intravaginal part of the

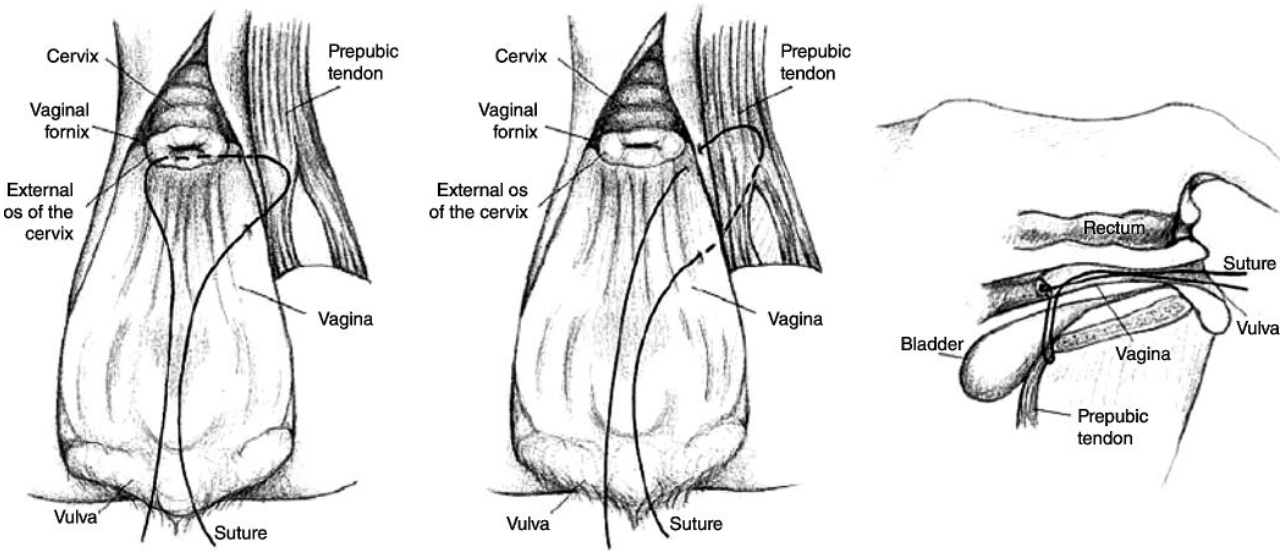


Figure 47.16 Schematic representation of Winkler technique for cervicopexy. *Source:* From Dean A. Hendrickson, A. N. Baird, Turner and McIlwraith's Techniques in Large Animal Surgery, © 2013, John Wiley & Sons.

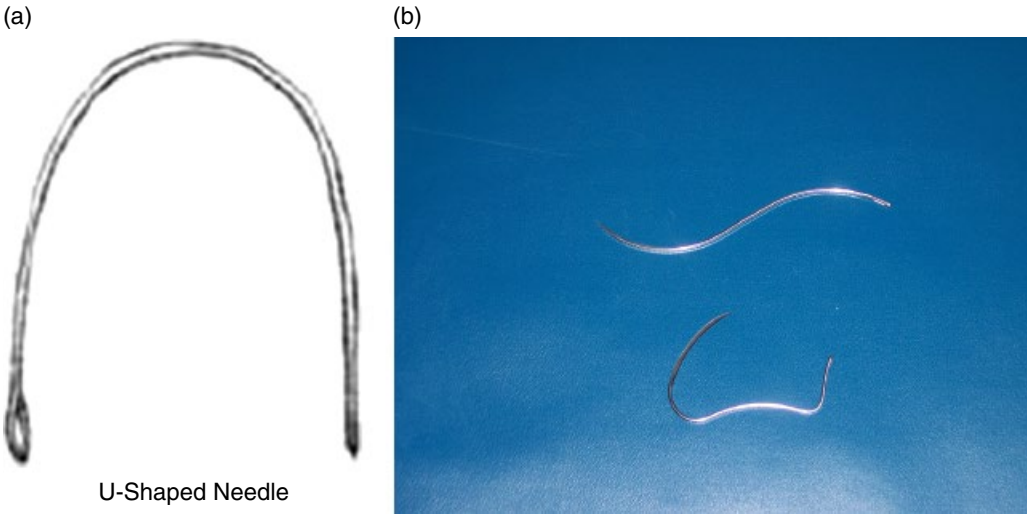


Figure 47.17 (a and b) Special needle used for Winkler technique for cervicopexy. *Source:* Illustration (a) by Dr. T. Barkley.

cervix; the needle is then directed across the lower half of the cervix and brought outside to tie a knot. This latter procedure may prevent breaking of the needle. A catheter is then passed through the urethra so that as the suture is tied the urethra is not trapped in the suture.

To tie a suture, knots are started on the outside of the vulva and slid forward. Suture is tied tight enough to prevent posterior movement of the cervix. To assist in placing cervical sutures, Knowles cervical forceps can be used to fix the cervix cranially, making passing of the needle through the cervix easier, or in some cows the cervix can be retracted sufficiently to be presented at the vulva again, making placing of the cervical sutures easier.

Other descriptions of this procedure do not advocate passing the needle through the cervical opening but rather through the side of the cervix without penetrating the lumen. This approach is greatly aided by fixation of the cervix with forceps [3, 26]. Advantages of this approach are that the cow can calve on its own, post-repair tenesmus is rare, and the cow can still be examined per vaginum. Occasionally the suture in the cervix can break, resulting in recurrence of eversion. This procedure is recommended for chronic cases which the owner wishes to keep, especially embryo donor cows, in which the problem is likely hormonally induced rather then there being any genetic predisposition. However, if the condition is likely to occur again or if it is probably hereditary in nature, the affected animal should be culled.

Uterine Eversion

Uterine eversion occurs mostly within 24 hours after fetal delivery, with the majority of cases occurring shortly after parturition. While relatively uncommon (<1% of parturitions) [35], it is nevertheless a true emergency situation encountered in practice. In many instances the everted organ is readily contaminated (Figure 47.18), traumatized physically or by extreme weather (heat/cold), becomes edematous, may hemorrhage, and may evert severely enough to rupture the uterine vessels, leading to rapid shock and death.

Owners should be instructed not to transport the animal and to place the everted organ inside a plastic bag to reduce contamination. Fortunately, many of these cows are unable to stand up and run due to hypocalcemia, partial paralysis, or exhaustion. However, on occasions, the cow is capable of standing up and may become fractious as she attempts to outrun the uterus or knocks the uterus between her hocks (Figure 47.19). Since these cases often result in rupture of uterine vessels and rapid death due to internal hemorrhage, one should approach them very cautiously.

Many factors [8, 36] are believed to contribute to uterine eversion, including reduced uterine contractility due to decreased uterine activity following primary or secondary uterine inertia. Hypocalcemia and reduced prostaglandin and oxytocin receptors and myometrial defects due to overstretching contribute to primary uterine inertia. Among others, mere physical exhaustion and dystocia are responsible for secondary uterine inertia. Apart from uterine inertia, situations in which there is continuing tenesmus as noted in dystocia, manual extraction of the calf, retained fetal membranes, delayed cervical involution, and laceration of the reproductive tract can lead to eversions. Prolonged recumbency that follows paralysis or hypocalcemia can lead to increases in intra-abdominal pressure, a potential factor for eversion. Hypocalcemia is also believed to make the uterus atonic, delaying the involution of the cervix, a predisposing factor for uterine eversion [37]. However, it has been suggested that hypocalcemia can be the result of uterine eversion rather



Figure 47.18 Everted uterus with evidence of contamination. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.



Figure 47.19 Uterus is further traumatized by knocking between the hocks. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

than a cause of it [38]. The roles of parity [39] and hypocalcemia still remain an enigma in uterine eversions etiology [40, 41]. Overconditioned animals may be more prone to increased intra-abdominal pressure due to increased fat reserves in the abdomen. Other non-animal factors can contribute to this condition. For example, outbreaks can occasionally occur due to extreme weather conditions and pasture composition. Further, it has been suggested that alterations in availability of dietary calcium, phosphorus, and magnesium may have a role in creating uterine inertia and predisposing animals to this condition. Uterine eversion outbreak noticed in animals kept on a cool-season grass pasture supports this hypothesis. It has also been suggested that high estrogen concentrations in pasture may play a role. The foregoing description undoubtedly attests to the multifactorial etiology of this condition.

Many methods of reduction and practical tips have been described [1, 36, 37, 42–60]. Replacing the uterus can be done in either a standing or a recumbent position, depending on circumstances and the clinician's preference. The animal may have to be cast, rolled, and pushed to lie on its abdomen to facilitate pulling the hind legs straight behind to facilitate reduction [36]. Obviously, if the cow is in pasture with partial paralysis, the recumbent position is dictated. Replacing the uterus in the recumbent position is greatly facilitated by positioning the cow in the sternal position with both hind legs extended out behind (Figure 47.20)

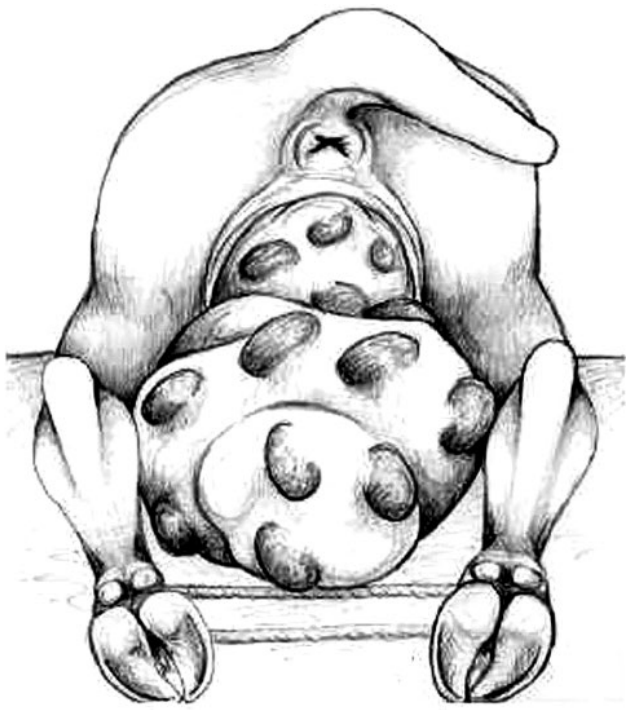


Figure 47.20 "Okie position:" note extension and elevation of hind legs. *Source:* Illustration by Dr. T. Barkley.

with the head facing downhill ("Okie position"). This position tilts the pelvis forward and allows the clinician to get behind the cow and cradle the uterus in their lap as it is placed back into the cow. Others prefer the standing position (Figure 47.21) if the cow can be adequately restrained and help is available to place a towel or bed sheet (or something similar) under the uterus and lift it up as the clinician replaces the uterus (Figure 47.22) back into the cow.

Replacement Guidelines

- Give epidural anesthesia.
- Remove as much fetal membranes as possible.
- Clean the endometrium.
- Placing the uterus in a plastic bag with cold water will assist in cleaning the uterus and reducing edema.
- A generous amount of obstetrical lube should be applied to assist in reduction.
- A pressure bandage can be applied for 10–15 minutes beginning distally and working toward the cow. Sugar may be applied sparingly. Use of hypertonic substances like granular sugar will further reduce edema but can cause more endometrial damage by drying and abrading the tissue. A 50% dextrose solution can be used to provide osmotic activity without mechanical irritation.
- Identify the non-gravid horn (strong intercornual ligament prevents its eversion) by observing for the presence of an oval or slit-like opening bordered by a ring of small cotyledons and reduce it first.
- Begin applying steady pressure at the cervix/uterine body area using both hands with a clenched fist to reduce the everted uterus into the vagina. Some clinicians prefer to wear oven mittens during reduction to avoid inadvertently puncturing the uterus with their fingers. Once the uterine body is reduced into the vagina, the uterine horns are gradually massaged into the birth canal or vagina.
- Fully invert the uterine horns. If the operator's arms are not long enough, the rounded bottom of a 1-l soda bottle, wiffle ball bat, or probang can be used to completely invert the uterus.
- Give non-steroidal anti-inflammatory drugs and parenteral antibiotics.
- Oxytocin should not be given until the uterus has been reduced or replaced.
- Use of a retention suture is discussed below.

A probang (Figure 47.23) is commonly used to make sure that the tip of the uterine horn is fully inverted. The rounded edges of a probang prevent possible tears that may occur to the friable uterine wall during the procedure. A child's wiffle bat can be used as an inexpensive alternative to the probang (Figure 47.24).

Failure to fully straighten the uterine horns can result in re-eversion or ischemic necrosis of a portion of the uterine horn. Placement of a suture in the vulva after uterine replacement is controversial. If the uterus responds to oxytocin and the cow is able to stand, then re-eversion is highly unlikely and a vulval stitch is not needed. On the other hand, if the cow is unable to rise and tenesmus recommences, a



Figure 47.21 Cow is placed in a chute (a squeeze chute is preferred) for standing reduction. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.



Figure 47.22 Elevation of uterus by a sheet helps in reduction. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.



Figure 47.23 A probang is used to fully invert uterine horns. Note that the ends are rounded. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

vulval stitch may be indicated for 24–48 hours. After reduction, 20–30 IU oxytocin should be injected and repeated two more times in 30-minute intervals and calcium therapy instituted if necessary. Antibiotic therapy is probably warranted in the case of a severely injured and contaminated uterus but may not be necessary in the case of a relatively fresh and cleanly reduced eversion.

Uterine Amputation

There are two approaches to uterine amputation: open and closed [1, 2, 61, 62]. The open approach involves incising the uterine body and location and ligation of the major uterine vessels in the stretched broad ligament prior to amputation of the uterus. An advantage of this approach is that any viscera contained within the prolapse can be returned to the abdomen before the uterus is removed. With this surgical approach, the wall of the uterine body is opened from the cranial cervix to bifurcation of the uterine horns to expose contents within the everted uterus and major vessels in the broad ligament (mesometrium). Vessels are identified by "fanning out" mesometrium and double ligated, preferably when they are not under tension. Vessels are severed between the ligatures and the mesometrium incised and allowed to retract into the abdominal cavity. A series of adjacent slightly overlapping mattress sutures are then placed across the uterine body just proximal to the cervix. The uterus is then amputated 2–3 cm below this line of sutures. The stump may then be sutured with a simple continuous pattern. Postoperative treatment should include administration of tetanus toxoid, non-steroidal anti-inflammatory drugs, and appropriate antibiotics.



Figure 47.24 A child's wiffle bat can be used in place of the probang.

In the closed approach (Figure 47.25) the entire organ is ligated and left to slough in 10–14 days. This is a salvage operation applied in cases of severe ischemic necrosis and/or lacerations. With this approach, viscera could be unintentionally trapped in the everted uterus with fatal consequences. Ligatures for this latter approach can be made of surgical tubing or bungee cord, or the bloodless castrating unit used for castrating large bulls works well.

Discussion

Vaginal and Cervical Eversions

Prepartum vaginal and cervical eversions can be perplexing and disturbing problems both to the producer and the veterinarian. Expense and effort can be costly at times. These everted structures should be promptly replaced within the

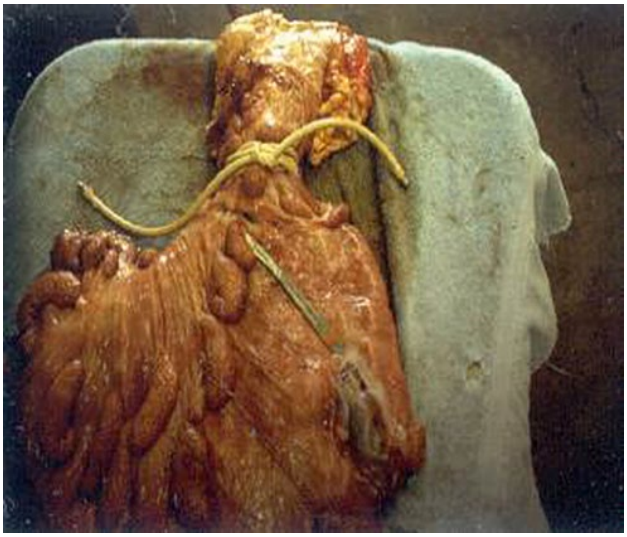


Figure 47.25 A closed approach of uterine amputation. Note location of ligation. *Source:* Reprinted from *Clinical Theriogenology* with editor's permission.

vaginal vault and secured such that contamination, laceration, sepsis, fibrosis, or necrosis do not occur. This approach will prevent compromise of cervical and vaginal relaxation at parturition and most importantly will enhance animal well-being by preventing unnecessary injury at parturition [63]. Some attempts made by producers to correct the situation can have many unintended consequences and can inflict unnecessary pain and injury to the animal. In this regard, client education is paramount. Although there may be temporary relief, the animal may evert again at the next parturition if the primary cause for an eversion is not corrected [11]. It is very important to realize that satisfactory reduction and retention alone are not sufficient since recurrence rate is high if the primary or inciting cause is not removed. This not only forces subsequent repair but more importantly, in some instances, can result in mutilation of vulval and perivulval tissue and become an important animal-welfare issue. Many techniques have been developed and stood the test of time. Each one is unique and the technique chosen depends on the condition and expertise of the clinician. What was said a few decades ago still stands true: better techniques for handling chronic vaginal eversions are needed [28]. New knowledge in this area by developing alternative techniques and improving current techniques will keep our discipline vibrant.

Not many studies have characterized the future fertility of affected animals. However, we ought to consider the possibility of future occurrence in light of animal well-being rather than fertility. In this regard, it is pertinent to consider some of the treatments administered. These are aimed at preventing recurrence by narrowing the birth canal [6, 21] by creating adhesions between the retroperitoneal surfaces of vaginal and adjacent or subjacent structures. These methods can prevent recurrence but may result in injury to the animal at parturition. Further, should these animals conceive, subsequent pregnancies are frequently complicated by recurrence. Hence, consideration should be given to culling affected animals after their calves are weaned [9]. Animal health advisors have an important role to play to help producers make a decision to eliminate these animals from the breeding herd. Considering the injury the animal may endure at the present and subsequent reductions, it behooves us to strongly suggest removing such animals from the herd [62].

Uterine Eversion

Appearance of a heavy mass of tissue outside the body of an animal can be intimidating and at times the process of reduction can be overwhelming. Truly the prospect of performing a successful reduction and the thought of recurrence can place one in mental anguish. We always look for ease of operation and reduced physical effort on our part to reduce eversion. In an attempt to facilitate the process of reduction, mechanical aids have been used successfully [49, 51, 57, 58]. However, raising the rear end of a live cow may appear inhumane [46]. Next, application of agents such as sugar [46, 59] or salt [53] on everted tissue to reduce edema before reduction is not supported uniformly. Although the authors prefer a careful application as supported by others in practice [46], it remains controversial [8] since it may amplify endometrial trauma. This technique may have certain academic skepticism despite its benefits.

The chance of immediate recurrence can be eliminated if reposition is done completely, without allowing invagination of the uterine horns to occur at the ovarian pole. Overall, the mortality rate ranges from 18 to 20% [8, 37]. Prognosis for survival and future reproductive performance depend on several factors, but time from occurrence to treatment is critical for a positive outcome. Despite extensive trauma that may occur before replacement, those that are replaced correctly and survive have an acceptable prognosis for future fertility [37, 64]. Occasional bladder eversion and intestinal entrapment that occurs with an everted uterus may result in a poor prognosis. Timely intervention by a professional can minimize animal discomfort and economic loss to the producer. Treatment given by an uninitiated non-professional has to be discouraged since severe damage can be inflicted to the animal [45]. A unique retention technique developed to prevent recurrence [60] should be considered to obviate situations such as necrosis, gangrene, and severe trauma, necessitating amputation.

The chance of recurrence at the next parturition has been discussed [2, 65] and heritability or additive susceptibility

with subsequent pregnancies for this condition is not apparent [1]. Since no known heritable component is recorded, there is no increased probability of occurrence at subsequent parturition. Therefore one should not be too hasty in recommending salvage of affected cows. Chance of recurrence is minimal and this has to be discussed with the producer. It should be remembered that in the developing world, cattle are an economic asset and cost of therapy must be balanced against the value of the animal. In addition to the value of the animal, its fertility will dictate whether the animal has to be culled or not. A major factor that can influence this decision is the degree of trauma caused to the uterus and the time it has taken to repair [36]. Future fertility of cows is reduced and there is a slight increase in risk at subsequent calving, but increased risk is not sufficient to justify culling [2].

As the profession moves more and more into animal health and production than restorative therapy, it is incumbent on us to prevent the occurrence of these conditions by careful selection of breeding stock, observing quality animal husbandry methods, providing adequate care to periparturient animals, and finally eliminating potential recurring cases. All the above will enhance animal well-being and place the profession as animal-welfare contributors.

Acknowledgments

Dr. T. Barkley of Woodward, Oklahoma created illustrations and her assistance is much appreciated. Figure 47.4 is reprinted from *Color Atlas of Diseases and Disorders of Cattle* by R. Blowey and A. Weaver with permission from Elsevier. Figure 47.16 is redrawn from *Techniques in Large Animal Surgery* by D. Hendrickson with permission from John Wiley & Sons. Remaining figures and content are reprinted from the *Clinical Theriogenology* journal with the editor's permission.

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48

Induction of Parturition and Abortion

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Introduction

Insight into the control of pregnancy and parturition began with the early work of Liggins [1, 2] and continues today with a great deal of research revolving around induction of parturition or abortion in cattle. This research has culminated in protocols for the induction of parturition and abortion that are successful and widely accepted in the veterinary and cattle industries today. However, these protocols are not perfect as the issue of retained fetal membranes following the induction of parturition is still a risk that one must consider. Another hurdle to the use of induction of parturition is the need for known breeding dates to ensure cows that are induced will give birth to a viable neonate. Current methods of estimating fetal maturity in the absence of known breeding dates are minimal and an area for further research.

Physiologic and Endocrine Profiles of Pregnancy

Progesterone is essential for the establishment and maintenance of pregnancy in all mammalian species that have been studied [3]. In cattle, the corpus luteum (CL) is the primary source of progesterone throughout gestation and luteal regression is necessary for parturition to occur [4]. Progesterone is also produced by the binucleate cells in the cotyledon of bovine placental tissue [5, 6]. This placental source of progesterone begins around 120 days of gestation. Between 120 and 150 days and approximately 240 days of gestation the placental source of progesterone alone is sufficient to maintain pregnancy. Placental progesterone gradually declines during the last four to six weeks of pregnancy and the maintenance of pregnancy once again becomes dependent on the CL [7, 8]. Thus if luteolysis occurred between the fifth and eighth month of gestation, pregnancy would be maintained for a time simply from placental progesterone, but parturition would likely occur several weeks before the normal due date once placental progesterone began to decline and the CL was expected to maintain pregnancy.

While the bovine placenta has the ability to produce progesterone, it does not contain the enzymatic activity for 17α-hyroxylase; therefore there is negligible placental estrogen production. However, as the fetal hypothalamus, pituitary, and adrenal glands mature, increasing amounts of cortisol are produced by the fetus. Rising fetal cortisol in the last four to six weeks of gestation gradually reduce uteroplacental synthesis of progesterone [9]. This decline in progesterone secretion is due to cortisol induction of enzymes that convert placental progesterone to estrogen. Cortisol induces 17α-hyroxylase and 17,20-desmolase activities in the placenta, catalyzing the formation of androgens from progesterone [10]. Androgens are then rapidly converted to estrogens by the placental P450 aromatase system (Figure 48.1).

During pregnancy, high concentrations of progesterone maintain uterine quiescence mainly by hyperpolarizing the myometrial cells. At the end of gestation, falling progesterone and increasing estrogen produces depolarization of the myometrial cells and stimulates the formation of myometrial cell gap junctions enhancing the sensitivity of the myometrium to stimulatory agonists [11]. Declining progesterone and rising estrogen results in increased expression of oxytocin receptors in the myometrium [12]. Increased estrogen concentrations also stimulate the production and release of prostaglandins [13]. Researchers have suggested that in sheep, fetal cortisol from the adrenals stimulates prostaglandin E2 (PGE2) synthesis in the cotyledons [14]. The increase in PGE2 results in increased upregulation of estrogen synthesis in the cotyledons which leads to the release of PGF2α

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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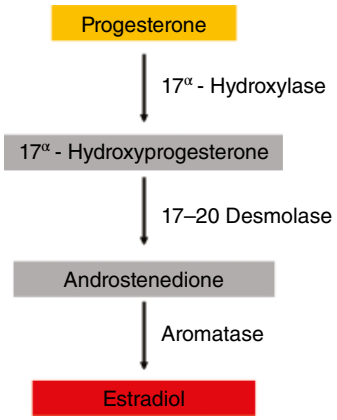


Figure 48.1 Progesterone is synthesized to estradiol following the upregulation of enzymes secondary to the rise in fetal cortisol.

from the endometrium, resulting in myometrium contractions. However, in cattle fetal cortisol directly induces PGF2α and PGE2 secretion in cotyledons [15–17]. Prostaglandins are also involved in the mechanisms of cervical ripening, which is essential for successful parturition.

Fetal endocrine control of parturition was demonstrated by Liggins in his studies on parturition in sheep. He showed that electrocoagulation of the fetal pituitary prevented normal term parturition and went on to demonstrate that the hypothalamus–pituitary–adrenal (HPA) axis was responsible for initiating parturition as infusion of glucocorticoid or adrenocorticotropin (ACTH) initiated premature parturition [18].

The trigger for the spontaneous onset of labor is the greatly increased cortisol production by the adrenal gland of the maturing fetus. The increase in activity of the fetal HPA axis at the end of gestation is likely caused by programmed maturation in the fetal hypothalamus, or elsewhere in the fetal nervous system, rather than a response of the fetal pituitary to a chronic stress such as might be caused by a developing inadequacy of space and nourishment within the uterus. Some other external influence on the maturing hypothalamus, such as a progressive increase in the secretion of the placenta, is another possibility [19].

Throughout the last two to three weeks of development in utero the fetal adrenal increases in size relative to body weight and the cellular sensitivity to ACTH increases [3]. The increase in sensitivity to ACTH is partially the result of increased adrenal cortical mass and partially the result of increased cellular responsiveness to ACTH. The increase in size and sensitivity of the adrenal gland combined with increasing circulation of ACTH account for the increased cortisol secretion that triggers parturition. Recent evidence suggests that at least some of the activity of the fetal HPA axis is due to positive feedback from placental estrogen. Increasing estrogen in fetal plasma greatly increases ACTH concentrations in fetal plasma [20].

Since fetal cortisol levels gradually rise in the last month of gestation, particularly in the last week [9], placental maturation may require exposure to elevated cortisol levels for a period of time prior to calving. This hypothesis is supported by a reduction in the incidence of retained placenta when long-acting corticosteroids are used to induce parturition [21, 22].

Induction of Parturition

Precautions

At present, one of the main obstacles to widespread use of induction of parturition is the need for accurate knowledge of breeding dates to prevent the birth of non-viable calves and an increased incidence of retained fetal membranes. Calves born up to two weeks preterm have good vitality and can attain good maternal immunity derived from colostrum [23, 24]. The incidence of retained fetal membranes is related to the degree of prematurity of the placenta at the time of induced parturition. Complete maturation of the bovine placenta occurs approximately three to five days prior to parturition [25, 26]. Rises in glucocorticoids and estrogen levels are crucial to the placental maturation process, with the increase in both hormones resulting from increasing fetal cortisol release during the last part of pregnancy [27–29]. Consequently, cows induced one to two weeks prematurely usually retain the placenta in over 75% of cases, whereas cows induced within a few days of term, or at term, have a 10–50% placental retention rate. In most reports, cows with induced parturition and retained fetal membranes did not have reduced pregnancy rates [30, 31]; however, first-service pregnancy rates were significantly lower in cows that had retained placentas than cows that did not retain their placentas [27, 32].

The effect of induction of parturition on milk yield in the following lactation and on the concentration and yield of colostral immunoglobulins most likely depends on how early parturition is induced. In general, when parturition is induced within two weeks of normal term, the onset of milk production would be a few days slower than in natural calvings, but overall production levels for the entire lactation period are within expected limits [9, 22]. When parturition was induced up to 22 days before the normally expected calving date there was a significant decrease in the yield (4–9%), but not the concentration, of colostral immunoglobulin G (IgG) [33]. When birth occurred 10–15 days or more before due date, calves had lower levels of colostral immunoglobulin [21, 33]. This may be due to

an impaired ability to absorb immunoglobulin [34, 35] and reduced intake due to calf weakness [21].

When short-acting corticosteroids are used to induce calving close to term, the immunoglobulin concentration of colostrum is not different from that in naturally calving cows; however, the total immunoglobulin content may be lower due to a reduced volume of colostral secretions [33, 34]. The ability of these calves to absorb immunoglobulin is not reduced, as serum γ-globulin concentrations after suckling are similar to those of naturally born calves [35, 36].

Indications

Induction of parturition has become increasingly more requested by beef cattle owners over the years. Especially, among those with cattle of significant economic value – whether that be the dam, fetus, or both, as seen in the seedstock and show cattle industries. Induction of parturition affords multiple advantages to the cattle owner, including concentration of labor, increased calvings during daylight hours due to the timing of injection protocols, and decreased chance of fetal oversize due to prolonged gestation length. These herds often have excellent breeding records, which include known breeding dates, artificial insemination dates, or embryo transfer dates, which are paramount in reducing known risks of inducing parturition which include non-viable, premature fetuses and retained fetal membranes.

Cows may be induced at 285 days of gestation to prevent prolonged gestational periods. This can be advantageous as the fetus can gain 0.45–0.68 kg/day in the final weeks in utero in normal gestations [37, 38]. When gestations are one to two weeks overdue, as is more commonly the case in some of the large European breeds of cattle, fetal weight gains could be as high as 1 kg/day [39, 40]. In such cases, calf birthweight might easily become too large to allow normal parturition. This is also the case with feti derived from *in vitro;* maturation (IVM)/*in vitro;* fertilization (IVF), which have been known to result in large offspring syndrome [41].

It has been reported that when a short-acting glucocorticoid was used to terminate prolonged gestation in continental breed heifers, there was dystocia due to poor birth canal relaxation and ineffective labor [36]. However, too early intervention in the calving process might have been partially to blame for this finding. In most cases, the stages of labor and pelvic relaxation are normal. Although no data are available, clinical experience from common usage of the procedure indicates that calf viability and colostral transfer are good. An additional benefit of induction of parturition in these cases might be to allow the female an extra 10–14 days to resume cycling for rebreeding in the subsequent breeding season. However, despite gestations having progressed for a normal duration, retained fetal membranes are a risk with induction of parturition. Thus the subsequent problem of metritis or endometritis may cancel any benefits of extra time gained through induction of parturition for the cow to resume cycling since endometritis may delay the first postpartum estrus.

The use of induction protocols in the commercial beef industry is often limited by the lack of known breeding dates and the inability to accurately determine fetal age in late gestation. Induction of parturition in commercial cows is often limited to the treatment of uterine hydrops, cardiac failure, or other health-related matters in which salvage of the fetus, or the life of the cow, is considered.

In dairy herds, parturition may be induced one to two weeks early to prevent excessive udder edema and distension, which may predispose the cow to mastitis and difficulty in milking. Induction of parturition with long-acting corticosteroids was once heavily utilized by dairy producers in New Zealand and Australia as an important management tool for initiation and synchronization of lactation with the grazing season [42, 43]. This management tool allows for a compact calving and consequently lactation season that allows for maximal utilization of the forage growing season. An additional benefit is that cows have the maximum number of days to become rebred in the abbreviated breeding season that follows. Inevitable though some cows will fail to become pregnant or will become pregnant too late to calve on time the following year. These cows would ordinarily be culled due to their inability to calve at the appropriate time to maximize days in lactation. Induced calving applied in association with an extended mating period reduces herd wastage. Cows that were not pregnant at the end of a defined mating season can be mated and identified by bulls fitted with a chin-ball marking harness. In the following calving season these cows can be induced to calve one to three months prematurely.

While the induction of parturition using long-acting corticosteroids has advantages from a seasonal dairy management standpoint, there are a number of downfalls including decreased calf viability, increased retained fetal membranes, photosensitization, infections, and cow mortality [44–46]. There are also mixed results regarding induction on lactational performance, with some studies reporting no effect and others suggesting a 4–9% reduction in milk yields [21, 47, 48]. Reproductive performance of induced cows does not appear to be substantially compromised in the subsequent breeding season despite increased rates of retained fetal membranes in multiple studies [21, 48, 49]. Due to animal welfare concerns the practice of induction of parturition in Australia and New Zealand has come under scrutiny. As a consequence, its use in herds has been dramatically reduced, especially in New Zealand dairy herds.

Methods of Induction of Parturition

Various types and combinations of hormone treatments have been studied for efficacy and safety of induction of parturition, including corticosteroids or prostaglandins in combination with various estrogen preparations [30, 31, 50–55] and oxytocin [56] without appreciably reducing the incidence of placental retention. Dimenhydrinate [57] and relaxin [58] in combination with dexamethasone have been reported to reduce placental retention; however, the number of cows treated with dimenhydrinate was small and relaxin is not commercially available. RU-486 or mifepristone, a potent antiprogesterone, used alone or in combination with relaxin and administered to beef cows on day 277 or 278 of gestation, resulted in basal levels of progesterone (P4) by 48 hours after injection and calving at 53–55 hours after treatment. There were no complications at calving and no retained fetal membranes; however, there were only two animals per treatment group and inductions were done very close to normal term, so a great deal more study is needed before this method can be recommended [59].

Short-Acting Corticosteroids

The most commonly used corticosteroids for inducing parturition are dexamethasone (20–30 mg) and flumethasone (8–10 mg) given as a single intramuscular injection. Parturition is induced with 80–90% efficacy when the injection is given within two weeks of normal term. The interval from injection to parturition is 24–72 hours, with an average of 48 hours. In cows that have not calved by 72 hours after treatment the induction is considered to have failed. Retreatment in such cases is often successful in inducing parturition. Relaxation of the pelvic ligaments, cervical dilation, and filling of the udder occur rapidly, and labor and parturition are normal. Calving difficulty scores that have been recorded for induced parturition have generally not been different from those of natural calvings; however, frequently a higher incidence of minor assistance is given. This is probably because personnel are readily available during induction trials and assistance is more likely to be given even when not absolutely necessary [60, 61].

Calves born less than two weeks prematurely are vigorous and calf mortality is not increased. The actual secretion of milk at the onset may not be plentiful in induced cows; however, colostral immunoglobulin levels and total milk production for the lactation period are very close to normal. Induced calves attain similar blood levels of immunoglobulins as calves born naturally. Retained fetal membranes were common with short-acting corticosteroids alone, with cows closer to their expected due (<1 week) dates having fewer reported cases of retained placentas compared to those that were further from due dates.

Long-Acting Corticosteroids

Long-acting corticosteroids alone allow for induction of parturition prior to expected due dates and in cases where calf viability is not of primary importance, as in the case of some seasonal dairy operations. Dexamethasone trimethylacetate (25 mg) or triamcinolone acetonide (TRI) (4–8 mg) may be used and appear to provide similar outcomes [22, 50, 62]. An intramuscular injection is given once 2–12 weeks prior to the due date for calving, and parturition occurs over a wide range of 4–26 days. Usually the further a cow is from her due date, the longer it takes for a response [43]. Despite prolonged elevated systemic corticosteroid levels, cow health is generally good; however, preexisting diseases, particularly subclinical infections, may be exacerbated by the treatment and there is a potential increase in cow mortality [63].

The udders of treated cows are consistently engorged with milk about one week after injection, although it may be another week or more before they actually calve. It has been suggested that these cows should be milked before calving if the udder is obviously full to prevent regression of secretory tissue. Total milk production per lactation can be expected to be reduced by 4–9% [21, 48, 49].

The incidence of retained placentas with the use of long-acting corticosteroids is quite low (9–22%) compared with short-acting corticosteroids. This is likely due to upregulation of placentome genes involved in PGE2 synthesis and the conversion of pregnenolone to androstenedione which facilitates functional maturation of the placenta [17]. However, there is a high incidence of calf mortality (7–45%), which appears to be due to calf prematurity, premature placental separation, and an increased frequency of uterine inertia.

The variability in response to treatment with a long-acting corticosteroid can be reduced by treating with a short-acting corticosteroid or prostaglandin about a week after the long-acting corticosteroid treatment [22]. Most cows will calve two to three days after the second injection. The interval from injection to calving tends to be shorter and more predictable after prostaglandin than after a short-acting corticosteroid, and fewer repeat injections are required with prostaglandins. However, calf mortality does not decrease with this method.

Prostaglandins

Induction of parturition with prostaglandins intramuscularly gives very similar results to induction with short-acting corticosteroids, with a range of 24–72 (mean 44.9) hours from treatment to calving. As with short-acting corticosteroid-induced parturitions, there is a high incidence of retained placentas and a 10–20% rate of induction failure when treatments are given within two weeks before normal term [64, 65]. The later the stage of gestation at

which prostaglandins are administered, the greater the efficacy with which a single injection induces calving.

Some investigators have reported that cows with prostaglandin-induced parturition, particularly those induced more than two weeks prematurely, suffer a higher incidence of dystocia due to uterine inertia or malpresentation of the fetus than naturally calving cows [66]. However, others indicated no difference in the frequency of dystocia in cows induced to calve one to two weeks prematurely with either corticosteroids or prostaglandins. Moreover, the induced parturitions had no greater frequency of dystocia than spontaneous parturitions. In one experiment it was observed that cows induced with cloprostenol had a longer interval from appearance of the placental membranes (onset of stage 2 of parturition) until delivery of the fetus than cows treated with dexamethasone or a combination of dexamethasone and cloprostenol. Cows receiving only cloprostenol had higher concentrations of progesterone at parturition, which may have suppressed uterine contractions compared with cows receiving dexamethasone. This may indicate that those receiving only cloprostenol may have had a placental source of progesterone that was eliminated in cows receiving dexamethasone [64].

Corticosteroids and Prostaglandins in Combination

Hormones used to induce parturition initiate endocrine events similar to those normally triggered by rising fetal cortisol levels. Corticosteroid injections appear to efficiently remove the placental source of progesterone by induction of enzymes that convert placental progesterone to estrogen. Failure of corticoids to induce parturition may be due to failure to remove the ovarian source of progesterone. On the other hand, prostaglandin injections are efficient removers of the ovarian source of progesterone, but may fail to induce parturition due to remaining placental progesterone. However, a combination of the two hormones acts in concert to remove both sources of progesterone, resulting in fewer induction failures and less variability in the time from treatment to parturition.

Experiments have been conducted to compare parturition induced by a combination of dexamethasone and cloprostenol, dexamethasone alone, and cloprostenol alone, with saline-treated controls [64]. Cows receiving a combination of prostaglandin and dexamethasone calved earlier (range 25–42 hours from treatment to calving), and the interval from injection to calving was less variable than with dexamethasone alone (29–65 hours) or cloprostenol alone (37–57 hours). In addition, with the combination treatment there were no induction failures, whereas dexamethasone and cloprostenol treatments resulted in induction failure in 10.5–16.6% [64]. The calving process for the combination treatment was considered to be normal. The rate of placental retention is significantly higher with all methods of induction of parturition than in control cows, but this was expected since with induction of parturition calving occurred before the due date and thus placental maturation would not have been completed.

Long-Acting Corticosteroids in Combination with Dexamethasone and Cloprostenol for Daylight Calving and a Low Incidence of Retained Placentas

At present, the main impediments to the use of induction of parturition are the need for known breeding dates, minimal control of the time of calving, and an elevated incidence of retained fetal membranes. It has been determined that calves that are born within the last two weeks of gestation are usually very vital and adjust well to extrauterine life [64].

Long-acting corticosteroids for induction of parturition resulted in the lowest incidence of retained fetal membranes compared with other induction methods. It would appear that the long-acting corticosteroids more closely mimicked the natural endocrine events of gradually rising fetal cortisol resulting in placental maturation. Cows receiving a combination of dexamethasone and cloprostenol had a less variable interval from injection to calving and there were no induction failures. Thus an induction regimen using a long-acting corticosteroid pretreatment followed by a combined dexamethasone and cloprostenol treatment was developed in hopes of establishing a protocol that results in reliable induction of parturition for calving during daylight hours combined with a low incidence of retained placentas.

Several experiments were carried out to determine the optimum dosages of two different long-acting corticosteroids and the optimum time interval from pretreatment with the long-acting corticosteroid and induction with combined dexamethasone–cloprostenol treatment [27, 32]. Experiments utilized Hereford and Hereford–Angus cows with known breeding dates. This important work is summarized here. In the first experiment [27], on day 270 of gestation 121 cows were placed in five groups. Groups I, II, and III received 25 mg of Opticortenol (dexamethasone trimethylacetate, a long-acting corticosteroid). Cows that had not calved by day 277 received 25 mg dexamethasone (DEX, Group II) or a combination of DEX and 500 μg cloprostenol (CLO) (DEX + CLO, Group III). Cows in Group IV received only DEX + CLO on day 277. Cows in Group 5 were untreated controls.

Pretreatment with Opticortenol followed by induction of parturition with DEX + CLO on day 277 resulted in calving during a very short time interval (28 ± 0.8 hours after induction) and the incidence of retained placentas was no different from that of the control group (13 vs 6%). The length of labor, birthweights, and calving difficulty scores were no different between groups. There was no difference in the

number of premature calves between groups and the only calf that died was in the control group.

In a second experiment [27], cows were assigned, in a 2 × 2 factorial design, to one of four treatment groups and a control group. On day 270 of gestation cows in treatment groups received either a high dose (1 mg/25 kg) or a low dose (1 mg/50 kg) of Opticortenol. The cows were further subdivided to be induced with DEX + CLO on day 274 or day 276. Induction treatments were performed at 9 a.m. in order to achieve daylight calvings. All cows in the treated groups calved between 7 a.m. and 11 p.m. about 30 hours after the induction treatment; however, the group receiving a high dose of Opticortenol and the induction treatment on day 276 had a lower incidence of retained placentas (29%) than other treatment groups. The incidence of retained placentas was 5% in the control group.

The length of stage 2 of labor, birthweights, calving difficulty, and calf viability were not different between groups and no premature calves were born in this experiment. Calf IgG concentrations at 48 hours of age were not different, whether the calves were born to induced cows or to untreated control cows, and there was no increase in neonatal illness in calves born to induced cows. In the breeding seasons following these experiments, the first-service conception rates and pregnancy rates did not differ between groups. However, first-service conception rate and overall pregnancy rate were lower in cows that retained their placentas than in cows that did not.

Two further experiments [32] were designed to determine whether pretreatment with a different long-acting corticosteroid, TRI, before induction of parturition with combined dexamethasone and cloprostenol (DEX + CLO) could be used to reduce the incidence of retained placenta and to produce a predictable calving time. The experiments were also designed to determine the optimum dosage of TRI and the optimum interval from pretreatment with TRI to induction treatment with DEX + CLO. It was determined that when cows are treated with TRI on day 270 of gestation, the optimum dosage of TRI was 1 mg/60 kg and the optimum interval from TRI to DEX + CLO was day 277. Cows receiving this combination of treatments had no retained placentas compared with 5% retained placentas in the control group. All induced cows calved between 24 and 48 hours after DEX + CLO, and 94% began to calve between 7 a.m. and 7 p.m., whereas only 58% of control cows began to calve during the same period of time. The length of stage 2 of labor, birthweights, calving difficulty, and calf viability did not differ among groups.

The results of these studies support the hypothesis that exposure to elevated blood corticosteroid levels prior to induction with DEX + CLO will result in a low incidence of retained placenta compared to induction with DEX + CLO alone. The use of long-acting corticosteroids to induce placental maturation followed by DEX + CLO to ensure parturition at a predictable time was quite successful in meeting the goals of calving in daylight, with a low incidence of retained placenta.

Induction of Abortion

Induction of abortion in cattle is a common procedure and is done for a variety of reasons. Heifer calves may become pregnant at a very early age, predisposing them to dystocia as well as retarded growth during pregnancy and during the subsequent lactation. Pregnancy in feedlot heifers usually results in reduced economic efficiency and calving management problems that feedlots are ill-suited to handle. Pathological conditions of pregnancy, including hydramnios, hydroallantois, fetal maceration, and fetal mummifications, must be terminated in order to save the life or breeding value of the affected animals.

Endocrinological Aspects

Pregnancy maintenance depends on continuous adequate concentrations of circulating blood progesterone whether pregnancy is normal or abnormal. All treatment methods intended to terminate pregnancy, except surgical removal of the fetus, must directly or indirectly eliminate the sources of progesterone. The CL is the main source of progesterone in cattle throughout pregnancy; however, the placenta and to a minor extent the adrenal glands contribute to circulating levels of progesterone. A functional CL is essential for the maintenance of pregnancy during the first 120–150 days of gestation and during the final month of gestation. From approximately 120–150 days and approximately 240 days of gestation placental progesterone alone is sufficient to maintain pregnancy; therefore lysis of the CL during this period will not terminate pregnancy.

Treatment of the pregnant bovine with prostaglandin results in luteolysis in both normal and abnormal gestations at any stage. In some cases, luteolysis appears to be incomplete and sufficient progesterone remains to maintain the pregnancy. In such cases, the female may undergo partial cervical dilation and may experience some abdominal straining, but then returns to the normal course of gestation and maintains the pregnancy to term. Ovulation and formation of a new CL may occur in these cases [65].

Glucocorticoids such as dexamethasone may reduce placental secretion of progesterone as early as the fifth month of gestation. However, glucocorticoid treatment alone will not induce abortion until the final months of gestation when these steroids are able to induce premature parturition.

In order for glucocorticoids to play a role in initiation of parturition, the fetoplacental unit must be functional. Thus in cases of fetal mummification or maceration, glucocorticoids will fail to induce abortion.

Methods of Inducing Abortion

Some of the older methods for the induction of abortions include per rectum enucleation of the CL, rupture of the fetal heart or fetal membranes and fetal decapitation, or the use of estrogenic hormones such as diethylstilbestrol or esters of estradiol. All of these methods have some significant disadvantages compared with prostaglandins [54, 67–70]. The luteolytic effect of prostaglandins will induce abortion during the first four to five months of gestation, which are dependent on the CL. During months five to eight of gestation, either PGF2α or glucocorticoid treatment alone will fail to induce parturition, although combined treatment with PGF2α or dexamethasone is highly reliable for inducing abortion during this period [71].

Situations Requiring Induced Abortion

Mismating

Veterinarians are sometimes asked to terminate pregnancy in a mismated female. The treatment of choice in these cases is to cause luteolysis. A luteolytic dose of prostaglandin may be administered as early as seven days after mismating since the bovine CL becomes responsive to prostaglandins by five to seven days after ovulation. Estrus will occur approximately three days after the prostaglandin injection and females can be rebred on that estrus if desired. First-service pregnancy rates will be normal on the estrus concurring with abortion for pregnancies advanced as far as 60 days at the time of prostaglandin-induced abortion.

Feedlot Heifers

Pregnant feedlot heifers are undesirable for a multitude of reasons, including problems associated with calving (an increased rate of dystocia, retained fetal membranes, and mortality), decreased average daily gain, inferior feed/gain ratio, and lower slaughter values [72–76]. Data from the National Animal Health Monitoring System (NAHMS) study suggest pregnancy rates in feedlot heifers are approximately 7–8%, but higher numbers may exist within some groups of cattle [77]. The main course of treatment is to utilize an abortifacient to terminate pregnancies early in the feeding period to minimize economic losses associated with heifers calving in a feedlot situation.

Heifers that calve or are aborted in the feedlot generally show poor performance in comparison with non-pregnant heifers. Problems associated with pregnant feedlot heifers include a lower feed efficiency and growth rate, lower dressing percentage and quality grades at slaughter, and management problems such as dystocia and retained fetal membranes should calving occur in the feedlot. It has been estimated that feedlot pregnancy results in losses of $44–115 USD per head [72]. The stage of gestation of the feedlot heifer will affect the degree of economic loss. In the early stages of pregnancy the anabolic effect of the luteal progesterone may actually be beneficial. Daily gains were higher in heifers pregnant up to 120 days and dressing percentage and quality grades at slaughter of heifers pregnant up to 120 days were equal to those of open heifers. Therefore heifers in early pregnancy and undergoing a relatively short feeding period could be expected to perform similarly to open heifers; however, this is seldom the case, with most pregnant heifers requiring four to six months on feed before slaughter.

All females that have recently entered a feedlot should be examined by transrectal palpation or ultrasound for pregnancy. Abortion can be induced at the time of examination. Animals induced to abort should be in good health and well adjusted to the feedlot, since induction of abortion may cause immunosuppression, especially if the protocol requires the use of dexamethasone, thereby increasing the disease risk of that animal. It is usually recommended that heifers be allowed a 21-day adjustment period to the feedlot before inducing abortion.

Growth-promoting agents containing progestins may interfere with induction of abortion and should be withheld until after abortion occurs. Heifers that are up to 150 days pregnant can be aborted with approximately 90% efficacy with a single luteolytic dose of prostaglandin [76, 78]. Those that are greater than 150 days pregnant must be treated with a combination of a luteolytic dose of prostaglandin and 25 mg of dexamethasone. Abortion will occur in 95% of these cases within 3–9 days.

Behavioral estrus occurs in 75–80% of aborting females, but since physical signs of abortion may go unnoticed and some fail to abort, all abortions should be recorded and the remainder of treated animals must be reexamined about 10–14 days after the abortion treatment. Lack of luteolysis is the most likely cause of abortion failure, and retreatment will usually induce abortion. Fetal mummification occurs in 2–4% of aborted females and this incidence is likely at least twice as high as that recorded in normal populations of cattle. Cattle that are more than four months pregnant at the time of abortion will retain the placenta in more than 80% of cases [13]. The placenta is usually passed in about one week and treatment is rarely necessary. Postpartum endometritis and purulent discharges occur in many heifers, but in most cases resolve spontaneously.

Ovariectomy as a means of inducing abortion and control of estrus in feedlot heifers has been suggested. In one experiment, ovariectomy was compared with manual or

chemical abortion and incorporation of melengestrol acetate (MGA) in the diet [79]. Ovariectomy reduced average daily gain and feed efficiency in the first 24 days. Prostaglandin-treated heifers reached slaughter condition earlier than ovariectomized or MGA-treated heifers. Only with the use of PGF2α were all pregnancies successfully terminated.

Hydropic Conditions of the Uterus

Hydropic conditions are seen sporadically in both beef and dairy cattle. Hydrops allantois is more commonly documented and can be a life-threatening disease for the affected animal due to the large volume of fluid that accumulates in the allantoic space. Salvage slaughter may be recommended, as prognosis for future fertility is poor to guarded. However, when treatment is selected, fluid drainage by abdominal catheterization paired with concurrent oral or intravenous fluid therapy should be considered as a first step in treatment. Large volumes of fluids will be required for several days to maintain hydration. Treatment to induce abortion with a luteolytic dose of prostaglandin in combination with 25 mg of dexamethasone usually will result in partial cervical dilation and accompanying loss of fetal fluid by 30 hours after treatment. Parturition is usually abnormal, with incomplete cervical dilation, primary uterine inertia, and lack of a strong abdominal press. However, since the fetus is usually small, with most cases developing at seven to nine months of gestation, fetal removal by repositioning and traction is usually successful. Postpartum metritis is a common sequela and frequently causes death. The prognosis for cesarean section is equally unfavorable because the uterus is atonic and friable and the fetal membranes are nearly always retained [80].

Fetal Mummification and Maceration

Fetal mummification or maceration occurs when a fetus dies without concomitant luteolysis and adequate cervical dilation to allow expulsion. A functional CL is often present and therefore the first step in treatment is the administration of a luteolytic dose of prostaglandin. Expulsion of the fetus usually begins within two to four days after treatment. A second treatment is usually not necessary. Estrogens have also been used; 50–80 mg of diethylstilbestrol or 5–10 mg of estradiol intramuscularly will usually result in fetal expulsion in 24–72 hours. (Editor's note: the use of estrogens in food animals is currently prohibited in the USA.) A second treatment may be necessary, and in rare cases three or four treatments at 48-hour intervals are required [80]. Whether prostaglandins or estrogens are used, expulsion of the fetus may not be complete because of poor cervical dilation and dryness of the cervix and birth canal. Lubrication and manual assistance are often necessary to achieve fetal delivery and postpartum medical care may be necessary.

Summary When deciding if and when to induce parturition, known breeding dates are a must if calf viability is of concern, and the risk of post-induction side effects including retained placentas and subsequent metritis should be discussed with the client. Long-acting corticosteroids followed by DEX + CLO have shown the most promise in controlling calving times and reducing the incidence of retained placentas but require more time and cattle to be handled multiple times. If inducing abortion, appropriate fetal aging is imperative to allow for the correct use of pharmaceutical agents, and pregnancies of 120 days of gestation or longer will require dexamethasone plus prostaglandin.

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49

Management to Prevent Dystocia

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Introduction

Dystocia is a term derived from Greek, meaning difficult birth, where the dam needs assistance in giving birth. It is well known that parturition is one of the most dangerous activities of any mammalian species and can result in the death of the dam, the fetus, or both. Thus, increased mortality and morbidity rates are to be expected in addition to labor and treatment costs. Even with management that one would determine to result in a successful outcome – an alive, healthy dam and calf – dystocia results have negative economic consequences. Following dystocia, a cow typically has a delayed return to estrus, which in turn impacts rebreeding in both beef [1] and dairy cows [2]. Also, calf performance has been shown to be negatively affected, with this being manifested by lower weaning weights.

The common etiologies for dystocia are fetopelvic disproportion, fetal malpresentation, a fetal abnormality, uterine inertia, and uterine torsion. Fetopelvic disproportion, which is simply described as excessive fetal size relative to the pelvic size of the dam, in addition to being the most common cause of dystocia [3], is realistically the only risk factor for dystocia that is amenable to preventive management and thus will be the focus of this chapter. Malpresentation as an etiology is typically overrepresented in the minds of cattlemen, as most forelimb and head malpresentations are in fact the result of fetopelvic disproportion.

Researchers have used kilograms of traction pressure or a more common five-point subjective scoring system to assign dystocia. The traction pressure can be measured with a traction pressure gauge attached to the calf puller [4]. The subjective calving difficulty scoring system (1–5) is determined as follows: 1 is designated for no assistance, 2 for easy pull, 3 for mechanical pull, 4 for hard mechanical pull, and 5 for cesarean section [4, 5].

Cursory thought on fetopelvic disproportion leads one to a couple of immediate areas to address: the increased incidence of dystocia in the heifer versus the multiparous female, and the impact of calf birth weight. Thus, heifer selection and development combined with management to avoid incompatible calf birth weights guide the approaches we will outline to decrease the incidence of dystocia.

Overall, studies show that it is paramount that producers have correct breeding management combined with genetic selection programs to decrease dystocia in cows [6]. Additionally, a timely diagnosis of dystocia and then assistance and correction will help reduce calf mortality [6].

Fetopelvic Disproportion

Fetopelvic disproportion results from the relative incompatibility of fetal birth weight and maternal pelvic size. Genetics, more specifically breed (strain within breed, and of sire and dam), influence calf birth weight, as do other factors such as gestation length, nutrition, season, and climate. Maternal pelvic size is influenced by parity, weight, frame (or skeletal size), and age.

Although birth weight and maternal pelvic size appear unrelated, there are factors that are associated genetically, such as frame size. This must be taken into consideration when management decisions are made to influence one without impacting the other. For example, when heifers in a commercial setting are selected solely based on pelvic area (pelvic height × pelvic width) there is a tendency to select older, larger heifers, which in turn typically have larger calves. Thus, an improvement in pelvic size is often

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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offset by having heavier calves. One must take all factors into consideration and use management tools in such a manner as to exploit each factor without detriment.

Risk Factors for Dystocia

The incidence of dystocia in heifers has been calculated to be anywhere from three to ten times that of multiparous cows, whether in beef [7] or dairy [8] cattle. This is consistent regardless of where in the world cattle are being raised [8]. Increased risk with heifer calving occurs when no interventional management is implemented.

In an observational study performed by Holm et al. [9], data were collected on a large group of heifers over two years. In addition to measuring body weight, pelvic area, and body condition score at the time of a prebreeding examination, the heifer's birth weight and the occurrence of dystocia at her birth was recorded. The heifer's body weight correlated to pelvic area, and for every increase of 1 cm^2 in pelvic area there was a 2% decreased risk for dystocia. Now one should note that this decrease in risk or odds of dystocia is not the same as a decrease in incidence. For example, if there is a 5% risk of dystocia, the odds would be 1 : 20 and a decrease in the odds of 2% would change that to 1 : 20.04. In this study, there was no association between dystocia and either the heifer's birth weight or dystocia occurring at her birth [9].

The birth weight of a calf is of course a central factor when addressing the issue of fetopelvic disproportion as a primary causative factor in dystocia. Additionally, calf birth weight represents an independent risk as the incidence of dystocia increases with every kilogram increase in calf birth weight in both beef [7] and dairy [10] cattle and this is independent of parity (Table 49.1). A recent study showed an increased incidence of dystocia in primiparous cows when compared to multiparous: 10.7 and 3.75%, respectively [6].

Table 49.1 Breed of sire means (in pounds) for 2011-born animals under conditions similar to US MARC.

Breed	Birth weight	Weaning weight	Yearling weight
Angus	87.3	577	1045.3
Hereford	91.7	571.5	1009.7
Red Angus	88.1	561.5	1013
Shorthorn	93.7	556.5	1022.9
South Devon	91.4	566	1030
Beefmaster	92.1	575.6	1002.9
Brahman	98.3	587.7	989.3
Brangus	90.8	568.2	1008.4
Santa Gertrudis	92.6	570.5	1013.9
Braunvieh	89.9	549.4	981.8
Charolais	94.7	592.4	1047.7
Chiangus	90.9	546.2	987
Chiangus	90.9	546.2	987
Gelbvieh	89.6	575.4	1027.1
Limousin	90.8	574.7	1007.7
Maine-Anjou	91.8	554.1	1000.8
Salers	89	566.4	1019.5
Simmental	91.5	586.1	1038.8
Tarentaise	89.1	576.2	1008.2

Source: Courtesy of Larry Kuehn, ARS, USDA.
Note: Because of the prevalent use of the English system of weights and measures in the calculation of EPDs, pounds were used in this table.

Heifer Management to Decrease Dystocia

Heifer calving has long been accepted as an inherent risk factor for dystocia. Efforts to address or ameliorate the incidence of dystocia in heifers focus on heifer selection and heifer development or conversely sire factors that would influence calf birth weight. For example, a study showed heifers had a higher dystocia rate when compared to cows [11]; more specifically, it has been shown that first parities are associated with 4.7 times higher odds for dystocia than later parities in dairy cows [10]. Another study compared beef heifers at two and three years old, with lower dystocia rates evident in the older heifers because of their larger precalving pelvic dimensions, with a consequent smaller disproportion between dam and calf sizes [4]. Also, primiparous cows were seen to be 2.5 times more likely to have dystocia when compared to multiparous cows [12].

Maturity index in heifers is calculated taking into consideration factors such as heifer age, birthweight, prebreeding weight, dam age, dam weight, and nutrition and it significantly predicted dystocia [13]. Thus, a higher maturity index was associated with decreased dystocia rate and also increased chance of a first and second pregnancy [13].

A usual practice is to breed heifers when they reach a target weight of 60% of their adult weight to minimize dystocia risks [5]. However, when heifers were bred at 53% of their mature birth weight there was no difference in dystocia rate when compared to heifers bred with 58% of their birth weight, which would reduce the costs of developing heifers [5].

Pelvic Measurement

Fetomaternal disproportion, with disproportional calf birthweight to dam size and conformation, will often result in dystocia. As pelvic conformation, narrowness of the pelvic

inlet, sacrum length, and skeletal maturity are primary maternal factors associated with dystocia, the assessment of pelvic measurement in heifers can be beneficial [14].

Measurement of the pelvic area is routinely done in beef heifers, using a caliper-type pelvimeter (Rice pelvimeter, Lane Manufacturing, Denver, CO; or a Krautmann-Litton Bovine Pelvic Meter, Jorgensen Laboratories, Loveland, CO), with pelvic height being the measurement between the cranial end of the pelvic symphysis and the dorsal wall of the pelvis, and pelvic width the measurement of widest distance between the medial aspect of the ileum. Thus, the pelvic area is calculated as the product of pelvic height × pelvic width [4, 9, 11]. Using the calf body weight at birth and pelvic area measurement, it was calculated that for a 1-kg increase in calf body weight there was a 37% increase in the odds of dystocia [4]. For every 1 cm^2 in pelvic area there was a 2% decrease in the odds of dystocia (after adjusting for calf body weight, year of birth, and dam parity) [4, 9]. Interestingly, neither the heifer's weight during her first calving nor the heifer's birth weight was associated with dystocia [4]. Additionally, it has been estimated that the odds of dystocia decrease by 11% per square decimeter (dm^2) increase in pelvic area of the dam [10].

In beef heifers, pelvic area affected dystocia; if heifers had smaller pelvic area measurements at 12 and 22 months (animals calved within 25 months) it resulted in more heifers needing cesarean sections [9]. Also, related to skeletal maturity of the dam, it has been shown that older heifers have a decreased risk of having dystocia even with primiparous cows having calves with lower birth weight and shorter gestational length when compared to multiparous cows [10].

When pelvic area results were used to estimate the culling of heifers, the remaining females had higher body weight and higher calving rate, with the calves showing a tendency to have higher birth weight [9]. Thus, this might be a good indication to measure pelvic area in heifers and use those results to decide if the animals should be rejected for breeding or bred with an easy-calving bull (one that sires calves with low birth weight) [14].

Furthermore, even though pelvic measurement in heifers has been shown to be a valuable tool in animal selection to decrease dystocia, it is rarely used in dairy cattle [15].

However, there are controversial results regarding the use of pelvimetry in beef heifers as it has been reported that pelvimetry was a bad test to predict dystocia, with a large overlap of pelvic area in heifers that had or did not have dystocia (heifers with both small and large pelvic area had dystocia) [11]. This negative outcome of pelvic measurements to predict dystocia might be due to imprecision of measurements with low agreement between and within veterinarians using the Rice and Krautmann-Litton pelvimeters [11].

Management that Addresses Calf Birth Weight

Sire Effects

Sire Breed

The sire breed is an important factor as it genetically and epigenetically contributes to fetal growth and calf birth weight. It is important to point out that maternal effects are less heritable than paternal effects [16]. A study using 4579 first-parity females (Angus, Hereford, or MARC II – one-fourth Angus, one-fourth Hereford, one-fourth Pinzgauer, and one-fourth Red Poll) mated to seven breeds of sires from the US Meat Animal Research Center (MARC) Germplasm Evaluation Program showed that Hereford, Angus, Gelbvieh, and Brangus sires resulted in calves least likely to result in dystocias [16]. Contrarily, Chiangus, Charolais, and Limousin sires were the most likely to sire calves that would need assistance at birth [16].

Expected progeny differences (EPDs) is a useful tool to identify sires that are likely to sire calves with low or high risk of dystocia. For instance, birth weight EPDs (BW EPDs) could predict the birth weight of the sire's calves in comparison to other sires. Moreover, calving ease EPDs (CE EPDs) and/or calving ease direct EPDs (CED EPDs) could predict the relative calving ease of a sire's calves in comparison to other sires. Therefore, in cases of heifers that are expected to be at high risk of dystocia, it is recommended to select sires with low BW EPDs or high CED EPDs to reduce the risk of dystocia [14].

Calf Birth Weight, Size, and Conformation

Calf birth weight is the key intermediate fetal factor affecting risk of dystocia, as heavier calves are often associated with larger body dimensions. It was estimated that an increase of 1 kg in birth weight corresponded to a 13% increase in the odds of dystocia in dairy cows [10]. Moreover, a study with beef heifers showed that calf birth weight was the most significant risk factor for dystocia occurrence and severity [4], with body weight explaining approximately 41% of the genetic differences in dystocia [16]. Again, breeding yearling heifers with sires with low BW EPDs should decrease the risk of dystocia.

Gestation length also influences calf weight, which is influenced by genetic effects [17]. It has been shown that cows that have a shorter gestation produce calves with lower birth weight. Therefore, to reduce incidence of dystocia, short gestation length can be genetically selected. Gestation length was positively correlated to both calf birth weight and dystocia in calves from 14 *Bos taurus* bulls of different breeds mated to Hereford and Angus cows [17].

Double-muscling, also known as muscular hypertrophy, is a genetic condition associated with muscular hypertrophy due to mutation of myostatin (MSTN, also known as growth differentiation factor 8, GDF8). Doubled-muscled calves are heavier and larger and consequently associated with a higher risk of dystocia. The highest frequency of doubled-muscled calves has been reported in Belgian Blue breeds [16]. Delivery by cesarean surgery is the best choice for such cases.

Fetal anomalies and monsters, such as conjoined twins, fetal duplication, schistosomes, and anasarca calves, are also a predisposing factor for dystocia.

Dam Effects

Dam Breed

The dam breed effects for calving difficulties were reduced for Simmental and Charolais and increased for Red Angus and Chiangus cows [16]. Additionally, it is important to point out that maternal effects are less heritable than paternal effects [16].

Moreover, it has been reported that dairy cows have a higher prevalence of dystocia when compared to beef cows, possible because beef calves have undergone a stricter selection for calving ease [6].

Nutrition and Body Condition Score

Body size and parity are the key intermediate maternal factors affecting the risk of dystocia. Females that have not received an adequate diet and have a low body score might have a predisposition for dystocia [21]. Inversely, if the cow has been overfed there usually is an increased calf birth weight and fat deposition in the pelvic area, consequently leading to dystocia [21].

Primiparous, two-year-old Angus heifers received different diets during the first trimester of pregnancy, with animals receiving a high- or low-gain diet so they had a different rate of liveweight gain. Although the treatments were successful, resulting in heifers with different weights between treatments, there were no differences between the treatments in the number of heifers that had calving difficulties that resulted in assisted parturitions, and calf birth weights were not different between the groups. Contradictorily, when different diets were implemented before insemination and during the first 90 days of gestation, birth weight of calves and postpartum weight of heifers were different; however, the small numbers were not sufficient to show whether the reduction of calf birth weight of 3–4 kg (8–15%) would be responsible for a reduction in dystocia [22].

Thus, it might be beneficial to restrict concentrate/energy intake and avoid heavy or poor feeding during the last three weeks of pregnancy to avoid fat deposition.

Effect of Calf Gender

Male fetuses are often associated with a longer gestational period and consequently heavier body weight than females. Therefore, male calves are often associated with increased risk of dystocia during parturition in comparison to female calves. It was estimated that male calves are associated with a 25% increase in the odds of dystocia in dairy cows compared to female calves [10, 15]. Another report shows that male calves were 9.3 times more likely to require assistance during parturition compared to female calves. Consequently, the use of sexed semen for female calves might be beneficial in reducing dystocia, especially in smaller yearling heifers [22].

Furthermore, various studies have shown that there was a significant greater calving difficulty with male calves [12, 14, 19, 20, 22].

Effect of Season

There have been reports showing the effect of season on birth weights and consequently dystocia in cows; however, there have been different results between studies. When calving during winter was compared to summer in Holstein cows, there was 15% higher odds for dystocia to occur during winter calving, possibly due to the fact that during cold winters there will be an increased uterine blood flow to the uterus, with a subsequent higher calf birth weight, and also because dams would consume more hay during cold temperatures, which would provide more nutrients to the fetus [4, 10, 15].

Also, related to effect of season, beef heifers that were bred at 15 months (using calving ease bulls) and calved as two-year-olds had increased blood flow, evaluated with Doppler ultrasound, at 210 days of gestation when they calved during the spring compared to fall and heifers with higher calf weight [23].

Fetal Malpresentation and Twining

The normal presentation, position, and posture consist of a calf in cranial longitudinal position, dorso-sacral, with extended limbs and neck. When calves are not in this manner, such as with a posterior presentation, there is an increased incidence for dystocia [21]. The incidence of malpresentations is 2.7–4.1% [24, 25]. This aspect is very important, as fetal malposition was reported to be the leading fetal cause of dystocia in a study conducted in Italy [6]. Therefore, it is recommended to observe heifers hourly

when calving starts and to be ready to assist if calving is prolonged or if the calf is not in normal presentation.

Twin calves usually weigh less than singletons [12]. However, twin calves are usually associated with higher malpresentations than single calves (17.4 vs 2.7%) [25]. Dairy cows carrying twins should be frequently observed during the prepartum period and assisted during calving [25].

Heterosis

Heterosis is the result of mating two diverse breeds of animals so that there is an interaction between genes that increase the performance of the resulting calf compared to the purebred calf [18]. This breeding practice should also result in more vitality in crossbred calves, which might result in lower dystocia incidence [19]. Also, breeding a dam and bull of different breeds might reduce calving difficulty [19]; however, results from controlled crossbreeding studies have varied [12, 19, 20].

When groups of crossbred cows, using pure Holstein dams and crossbred dams of Holstein with Normande, Montbeliarde, and Scandinavian Red, were bred to Brown Swiss, Montbeliarde, and Scandinavian Red bulls the cows with crossbred calves had less calving difficulty when compared to those with purebred calves [19].

Conversely, a study that collected data during approximately four years with the objective of evaluating the percentage of pregnancy parameters when Holstein and Jersey animals were crossbred, with special consideration to avoid inbreeding in the purebred crossings, showed that direct heterosis was not significant for incidence of dystocia; however, there were direct genetic effects with purebred Holstein calves having more chances of requiring assistance at birth. Even with no statistical difference with crossbreeding, this study concluded that it is beneficial to use a Jersey sire on a Holstein dam to minimize dystocia [12]. Another study evaluated nine different breeds that were bred to generate three composite populations for effects of heterosis and found that although it was significant for calf birth weight, it was not significant for calving difficulty [20].

Overall, these studies suggest that there will be lower dystocia incidence if producers use bulls and cows of different breeds to produce crossbred calves.

Genetic Effects

With the use of high-throughput screening, such as next-generation sequencing (NGS), additional genetic information can be established for cattle regarding calving traits, including calving ease which is directly related to incidence of dystocia. One approach that has been used is to analyze the quantitative trait locus (QTL), which corresponds to a region of DNA associated with a specific phenotypic trait, to identify genes that have an importance for calving traits. Additionally, single-nucleotide polymorphisms (SNPs) consist of a variation at a single position in a DNA sequence of an individual, located in specific genes, and are identified to be associated with calving traits.

A study using Illumina paired-end sequencing examined QTLs and defined a region that was related to calving ease targeting the BTA18: 57 321 450–57 625 355 region of the *B. taurus* genome that had been previously identified as having a strong effect for calving traits. This work was able to identify candidate genes located in the BTA18 gene cluster that were associated with calving ease, such as sialic acid-binding immunoglobulin (Ig)-like lectin 12 (SIGLEC12), CD33 antigen-like (CD33), and carcinoembryonic antigen-related cell adhesion molecule 18 (CEACAM18) [26].

Assisted Reproductive Technology (ART)

The use of assisted reproductive technology (ART) in cattle has increased since the 2010s. However, one of the disadvantages of this technology is that some embryos derived from ART have abnormal development or developmental anomalies such as large offspring syndrome (LOS). The term LOS is used to describe calves born as a result of somatic cell nuclear transfer (SCNT) or *in vitro* embryo production (IVP). LOS is characterized by the birth of calves with heavier birth weight than normal. A study showed that 26% (of 27 calves) were above the 97th percentile of body weight, showing the overgrown feature often associated with LOS after ART [27]. Thus, with these larger calves there is an increased risk of dystocia, necessitating assistance during calving.

Summary

In a well-managed herd which has proper management at artificial insemination/natural service, before calving and at calving, considering factors such as when to breed heifers, proper sire selection, proper nutrition, and having trained personel to assist calving, the percentage of births that need assistance is expected to be low. As dystocia can result in costs of assistance and veterinary service or death of the calf and dam, it is very undesirable for it to occur in a cattle breeding operation (Figures 49.1 and 49.2).

Therefore studies using large numbers of animals, modern statistical models (such as QTL), and high-throughput sequencing are essential to better define risk factors that can lead to calving difficulty, so that veterinarians and producers can better manage beef and dairy cows and reduce dystocia rates in herds.

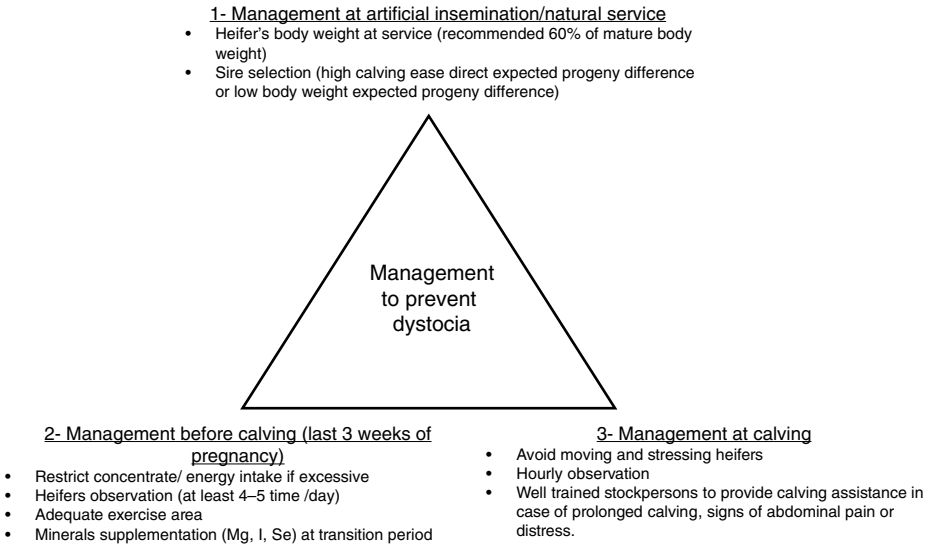


Figure 49.1 Management strategies to prevent dystocia. Mg, Magnesium; I, iodine; Se, selenium.

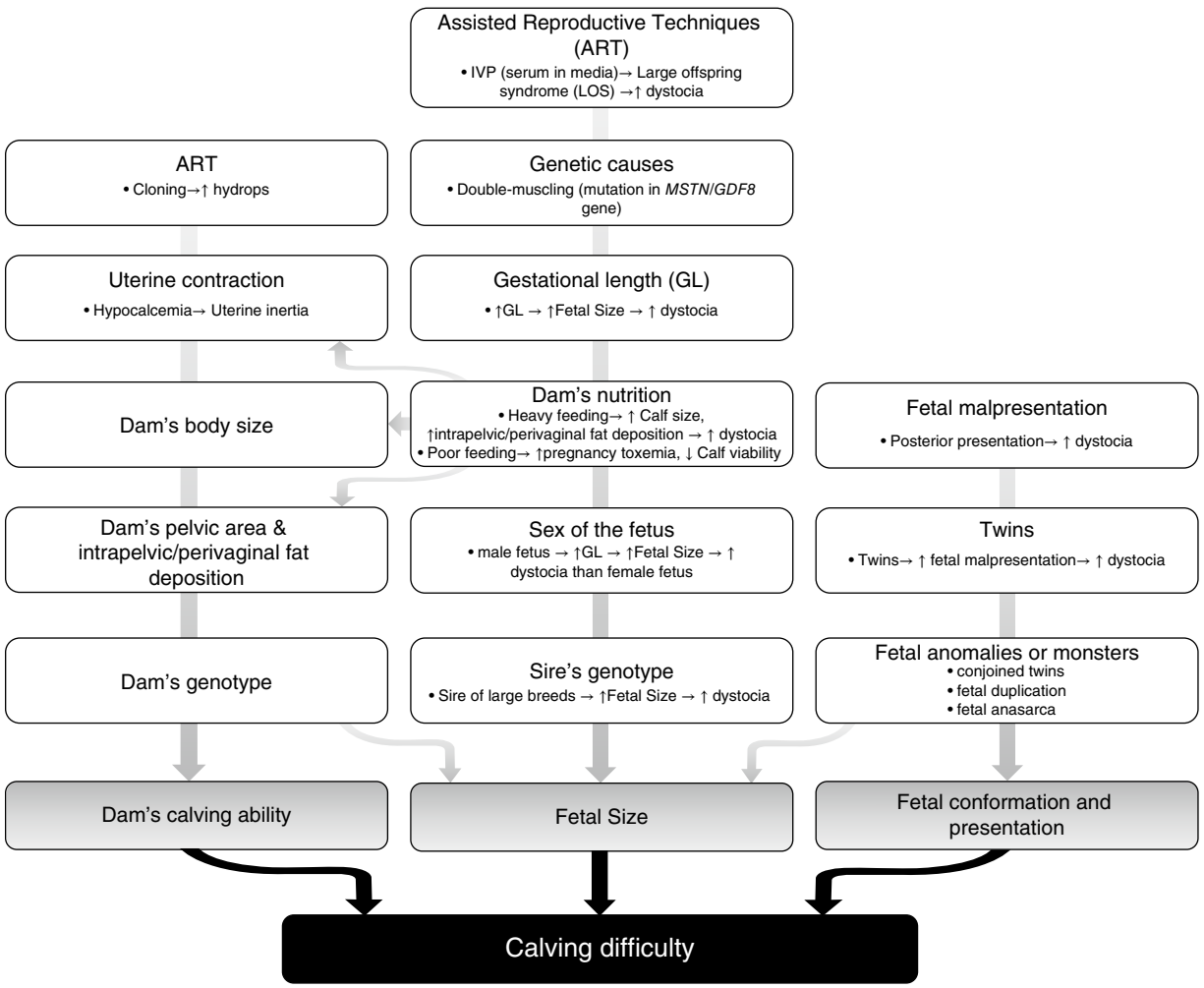


Figure 49.2 Risk factors affecting calving difficulties. ↑, Increase; ↓, decrease; *MSTN/GDF8,* (myostatin).

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Dystocia and Accidents of Gestation

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Introduction

To produce saleable products, replenish herd populations, and align the genetic base with production goals, sustainable cattle production relies on successful reproduction. The establishment and maintenance of pregnancy requires the integration of a complex series of events with many opportunities for disruption that may compromise gestational outcome, including failure of fertilization, embryonic death, abortion, pregnancy-associated disease of the dam, birth of animals with congenital anomalies, and perinatal death losses. Intricately choreographed events leading to successful gestation are sometimes imperfect and accidents of gestation adversely impact reproductive success.

Embryonic Death

Following appropriately timed insemination with high quality semen, fertilization (i.e. union of male and female gametes to form a zygote) rates are high in otherwise healthy and anatomically normal cattle [1]. Failure of the development of the embryo to the fetal stage (42 days in cattle) is termed embryonic death, which is often arbitrarily subdivided into early embryonic death and embryonic death later in development, without a universally accepted definition of the stage of development separating the two. The corpus luteum undergoes luteolysis following embryonic death and, though frequently unobserved, the products of conception are expelled from the uterus via the cervix (Figure 50.1) [2]. Although abortion and perinatal death are easily recognized as sources of reproductive failure, embryonic death is the larger component of overall pregnancy wastage.

Strategies to minimize embryonic loss have proven elusive but are emerging with investigations of the causes of failure of pregnancy to develop. Several review articles [3–5] outline specific conditions associated with embryonic death and pregnancy wastage, including uterine environmental deficiencies, heat stress, teratogenic toxins, nutritional deficiencies, viral infections, and chromosomal abnormalities. Characterization of the bovine genome has facilitated discovery of genetic combinations which are embryo lethal in the homozygous state [6–9] and discovery of other embryo lethal mutations is expected to follow.

Development of commercially available genomic testing provides a viable method to identify carrier animals for recognized conditions. Breed associations working with progressive producers are actively implementing strategies to minimize reproductive loss as these tools become available.

Fetal Death

Abortion

In cattle, a conceptus greater than 42 days gestational age is defined as a fetus, and pregnancy loss after onset of the fetal period and prior to the possibility of extra-uterine survival is appropriately referred to as abortion. Of lesser overall magnitude than embryonic death, abortion results in loss of an annual production cycle for the cow and frequently results in culling.

Abortion may be the result of bacterial, viral, or fungal infections, toxins, genetic defects, nutritional imbalance or deficiency, endocrinopathies, stressful events, or inappropriate pharmacologic intervention. Obtaining an accurate diagnosis is necessary to formulate and implement effective preventive measures and requires the investment of time and resources. Unfortunately, even with extensive

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine



Figure 50.1 Embryo and associated embryonic membranes recovered from the vagina of a cow accidently administered prostaglandin F2α.

diagnostic investigation, the etiology of abortion may not be apparent and the proportion of case submissions successfully diagnosed at accredited diagnostic facilities seldom exceeds 50%. Laboratory diagnostic success improves with an accurate herd history and timely evaluation of appropriately collected samples, including the fetus and placenta, and maternal serum, and with multiple case submissions when more than one pregnancy loss occurs.

Fetal Mummification

Following death in utero, the bovine fetus is usually expelled from the uterus and passes through the cervix and vaginal vault. Retention for up to a day or longer resulting in autolysis prior to expulsion is not uncommon. Occasionally expulsion efforts fail and the dead fetus is retained within the uterus. If luteolysis does not occur and the retained fetus is not contaminated by bacteria, the cervix remains closed and the conceptus can undergo an aseptic process of partial desiccation and absorption of much of the fetal and placental fluids, leading to fetal mummification [10]. Mummification following fetal demise occurs in only a small percentage of bovine abortions. Most cases of fetal death resulting in a mummified fetus occur between the third and sixth month of gestation, with occasional reports in more advanced pregnancies. Cows carrying mummified fetuses exhibit no obvious external signs other than anestrus associated with the retention of the corpus luteum and vaginal discharge is not seen until the cervix opens following luteolysis.

Diagnosis is usually made at the time of routine examination for pregnancy or during examinations prompted by failure to calve at the expected time. Transrectal palpation of the uterine contents is generally sufficient for diagnosis, with the finding of a relatively thick-walled uterus tightly surrounding the fetal remnants. Palpable placentomes are absent and the contours of the fetus are smooth, rather than irregular and sharp as occurs in cases of fetal maceration. Transrectal ultrasound examination of the uterine contents is a useful diagnostic aid.

Retention of the mummified fetus may resolve following spontaneous lysis of the persistent corpus luteum and the expulsion of the mummified remnants into the vaginal cavity. The passage of the mummy may be the first indication of pregnancy loss [11]. Often the cow will be observed straining when the fetus enters the vaginal vault and complete resolution may require manually assisted vaginal retrieval of the fetal remnant.

Induction of luteolysis with prostaglandin F2α is the most common therapeutic intervention following diagnosis of fetal mummification. The cow or heifer should be monitored daily for complete expulsion of the fetal remnants and assistance provided if necessary [10]. Multiple treatments may be required in some cases. Successful rebreeding following either spontaneous or induced expulsion of a mummified bovine fetus is common.

In some cases, luteolytic therapy fails to result in expulsion of the mummified fetal remains. Surgical removal via hysterotomy may be required for refractory cases and for larger fetuses. Surgical retrieval may improve the preservation of fertility compared to assisted transcervical extraction [12]. Surgical approaches that provide access to the uterus through flank laparotomy, ventral midline incision, or colpotomy are described in Chapter 55 of this text. The choice of surgical approach is guided by the surgeon's experience and the size of the mummified fetus (Figure 50.2).

Fetal Maceration

In the absence of a functional corpus luteum, aborted fetuses are usually expelled following fetal death but may be retained when cervical dilation is insufficient to allow eviction of the fetus. A retained aborted fetus will initially undergo autolysis. Degeneration is accelerated as fetal tissues are contaminated with either abortifacient bacteria or saprophytic bacteria which gain access to the uterus through the open cervix. At normal body temperature, bacterial contaminants invade fetal tissues and multiply rapidly. If not expelled in a timely manner, fetal soft tissues will progressively undergo a process of degeneration and liquefaction known as fetal maceration, leaving shreds of fetid fetal tissue, skeletal remnants, and inflammatory exudates within the lumen of the inflamed uterus.

The physical condition of cows with a macerated fetus in utero can range from severe systemic illness associated with overwhelming metritis and toxemia, to cases in which

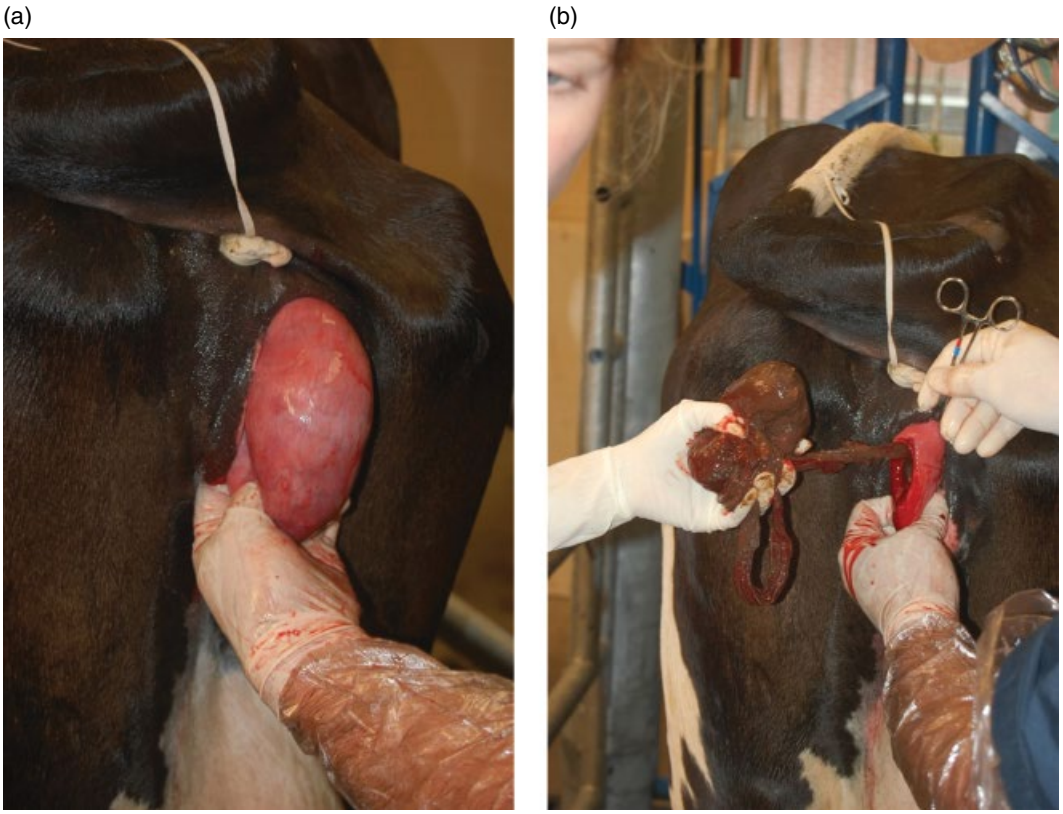


Figure 50.2 (a) Uterine horn containing a fetal mummy exteriorized through the vulvar lips utilizing a colpotomy incision. (b) Extraction of a fetal mummy from the uterine horn exteriorized through the vulvar lips utilizing a colpotomy incision.

intermittent straining and the presence of a fetid vaginal discharge in the dam are the only readily apparent signs. In some cases, all signs of systemic disease in the dam may have resolved by the time of examination, but the cervix will remain at least partially open and a fetid uterine discharge from the vagina is present. Diagnosis is based on physical findings utilizing transrectal or transvaginal palpation of the uterine contents. In contrast to the rather smooth contours of a mummified fetus, maceration-associated degeneration results in skeletal collapse and formation of sharp bony fragments, with a palpable crepitation of the disjointed skeletal remnants described as feeling similar to a bag of gravel. If no systemic disease is present, salvage by slaughter is recommended. Humane euthanasia should be considered for many other cases.

Manual dilation of the cervix and piece by piece removal of degenerating fetal remnants is possible but is difficult and time consuming. Bony fragments are embedded in the wall of the tightly contracted and markedly inflamed uterus (Figure 50.3). Options for surgical retrieval are hampered by the lack of an approach that provides sufficient access to exteriorize the tightly contracted uterus, and the presence of septic uterine contents. Following successful manual removal of fetal remnants and appropriate supportive care, some cows improve and gain weight but rarely achieve a subsequent pregnancy.

Developmental Defects and Congenital Anomalies Resulting in Fetal Death or Production of a Non-Viable Neonate

Successful pregnancy results from a complex series of developmental events which culminate in birth of a normal neonate. Deviations from normal development can result in pregnancy wastage in the embryonic or fetal stages or in stillbirth, birth of non-viable neonates, or birth of defective but viable offspring. Defects present at the time of birth are classified as congenital, with their origins predating parturition, and result from disruptions in normal developmental sequences. Viable neonates with congenital defects are adapted survivors from single or multiple disruptive events which occurred during fetal or embryonic development. Similar disruptions can result in fetal death (abortion) in those fetuses that cannot compensate for loss of function (Figure 50.4).



Figure 50.3 Skeletal remnants from macerated twin fetuses extracted per vagina. Removal of a macerated fetus through the partially open cervix is difficult and smaller bones are tightly embedded in the endometrium.



Figure 50.4 Duplication of the fetal head and neck. Fetuses with duplications of the head or limbs (dicephalus or polymelia) do not conform to the limits imposed by the birth canal and result in dystocia. *Source:* Image courtesy of Dr. Ethan Andress and Dr. Jenna Innes, West River Veterinary Clinic, Hettinger, ND.

Historically, the origins of most developmental disruptions were undefined and the resulting offspring were described as the result of accidents of gestation. Severely malformed cases were referred to as fetal monsters. More recently, genetic, toxic, and infectious agents have been causally associated with several specific syndromes and over time definitive causes of many other recognized congenital anomalies will be discovered, providing opportunity for effective avoidance strategies. Development of commercially available genetic testing services allows breed associations to address the incidence of specific recognized hereditary mutations by either prohibiting inclusion of carrier animals in the respective herd book or identification of carriers to minimize occurrence of affected individuals through informed breeding management decisions.

Non-genetic etiologies for congenital anomalies include toxins and viral agents. Teratogenic toxins found in plants or introduced into the environment in the form of synthetic chemicals are implicated as agents inducing congenital anomalies. Nearly identical anomalies may occur following infection of the pregnant cow with any of several viral pathogens coincident with fetal development in the late embryonic and early fetal periods. Avoiding exposure to potentially teratogenic plants, toxins, and viruses during critical periods of fetal development and enforcement of biosecurity and vaccination protocols are necessary components of overall reproductive management. Often the cause of fetal malformation is not known. *Schistosomus reflexus* (Figure 50.5) and *Perosomus elumbis* are two well-recognized instances of fatal fetal deformity for which a definitive cause has not yet been discovered.

Severely deformed calves will not readily conform to the physical limitations imposed by the birth canal at parturition and result in memorable dystocias necessitating fetotomy or cesarean section.

Vaginal Prolapse

Eversion of the vaginal floor or wall and/or of the cervix through the vulvar lips resulting in vaginal or cervicovaginal prolapse occurs most commonly during mid to late



Figure 50.5 *Schistosomus reflexus* fetal monster. Arthrogrypotic limbs and abnormalities of the axial skeleton make vaginal delivery of severely malformed fetuses difficult. Delivery by cesarean section or fetotomy is usually required.



Figure 50.6 Vaginal prolapse with eversion of vaginal wall and external cervical os.



Figure 50.7 Reduction of prolapsed vaginal tissue following cleansing and debridement.

gestation. Increased abdominal pressure from the presence of the large gravid uterus, a physiological increase in circulating estrogen concentrations during advanced pregnancy, and anomalies of the vaginal wall and cervix leading to tenesmus are contributing factors to subsequent eversion of the vaginal mucosa. Vaginal and cervical trauma associated with previous parturition or dystocia and excessive abdominal fill associated with diets composed of low quality forage may also play a role. In some breeds (Hereford and Shorthorn) primary vaginal prolapse exhibits familial patterns, suggesting a heritable phenotypic component. *Bos indicus* cattle and their crosses often have a posterior widening of the caudal cervical rings with a large external cervical os that allows eversion of more cranial cervical rings following successive parturitions and contributes to the occurrence of cervicovaginal prolapse in those breeds. Pregnancy-associated vaginal prolapse is likely to recur in subsequent pregnancies and subsequent breeding of affected animals is not recommended. Similar vaginal prolapses are sometimes seen in genetically valuable animals subjected to repeated superovulation for collection of embryos and are not thought to be heritable.

Diagnosis of primary vaginal or cervicovaginal prolapse is based on the typical appearance (Figure 50.6). Initially the vaginal mucosa may only be seen when the cow is in sternal recumbency, but a progressive cycle of irritation, tenesmus, and accumulation of tissue edema eventually results in more permanent protrusion of the vaginal floor and sometimes the external cervical os. Treatment consists of appropriate debridement of damaged tissues and reduction of the everted vaginal tissues into the pelvis, reestablishing anatomy of the vagina (Figure 50.7).

Desiccation and trauma to the exteriorized vaginal mucosa accumulate over time and complicate reduction of the prolapsed tissues in long-standing cases. A variety of retention techniques described to maintain the tissues in normal alignment following reduction are discussed Chapter 47. The practitioner's choice of technique must always include an assessment of the proximity to calving and the impediments to successful parturition inherent in many of the retention strategies.

Uterine Torsion

Uterine torsion is an infrequent accident of gestation and results in dystocia requiring specific obstetrical interventions or cesarean section. Both the degree of uterine torsion

and the duration of the torsion affect progression of labor, clinical outcome, and case management [13]. Uterine torsion can occur in any breed, with large, deep-bodied cattle and dairy breeds having increased risk. Brown Swiss cattle are overrepresented and *B. indicus* breeds may have a lower incidence of occurrence, perhaps due to lower birth weights and anatomical differences in the attachments of the broad ligaments to the uterus. Predisposing factors include a previous pregnancy and the presence of a large fetus [14]. Twin pregnancies are less likely to be affected [15].

Not all factors contributing to torsion of the uterus are understood. The uterine horns are suspended from the body wall by the broad ligaments which attach on the ventral surface and lesser curvature of the uterine horns. As pregnancy progresses, much of the enlarged uterine horn containing the fetus extends cranial to the area of abdominal attachment of the ipsilateral broad ligament and occupies the ventral abdomen, stabilized by the proximity to the body wall, the rumen, and other abdominal viscera. Because cattle typically rise from recumbency by first elevating the rear quarters, and assume recumbency by first lowering the fore quarters and cranial portion of the body, the pregnant uterus will be suspended by the broad ligament multiple times daily as the cow lies down to rest and arises from sternal recumbency, providing an opportunity for rotation of the gravid uterine horn following a slip or fall, or as the result of unfortunately timed fetal movements [14]. Other contributing factors may include a lack of rumen fill and poor abdominal muscle tone.

Most diagnosed cases of uterine torsion in cattle are late gestational events and are frequently first recognized at the time of obstetrical examination following failure of parturition to progress normally. Torsions of 45–90° are sometimes discovered in otherwise healthy cattle examined transrectally for pregnancy diagnosis or other reasons and often resolve without intervention.

Torsion of the uterus up to 180° does not result in vascular compromise and clinical signs do not become evident until the onset of labor and failure to progress toward delivery. As the degree of torsion exceeds 180°, uterine blood flow is increasingly compromised and subsequent venous congestion is followed by uterine and placental edema which may lead to death of the fetus. Ischemic necrosis of the uterine wall may follow and predispose to uterine rupture and death of the dam.

Successful manipulation to correct torsion of the uterus requires assessment of the direction of the torsion. When diagnosed at term during an obstetrical examination, a spiral fold in the floor and wall of the vagina is often palpable and allows the obstetrician to recognize both the presence and direction of the torsion. When the vaginal examination does not clearly define the direction of the torsion, as can occur in preterm torsion or in a torsion that has no vaginal involvement, transrectal palpation of the broad ligaments of the uterus allows determination of the direction and degree of twisting and facilitates case management. The normal relationships of the broad ligaments and uterus and the altered relationships in clockwise and counterclockwise torsion of the uterus are illustrated in Figure 50.8.

Dystocia due to uterine torsion when the cervix is dilated and the degree of torsion does not prevent manual access to the fetus per vagina can often be addressed by direct manipulation of the fetus and uterus to reestablish the normal anatomic relationships. If the operator's hand can be placed on the head or shoulder of the calf, the torsion can often be relieved by repeatedly rocking the fetus dorsally in ever-increasing arcs opposite the direction of the torsion until the center of gravity of the pregnant horn passes the critical point of balance and the uterus settles into its normal location. This technique requires considerable physical strength and is most successful when the operator is of sufficient stature to gain the necessary mechanical advantage during the manipulation.

If direct manual manipulation and rocking of the fetus is not feasible, use of a detorsion rod in conjunction with obstetrical chains is equally beneficial in cases with vaginal access to the fetus. Figure 50.9 illustrates a uterine detorsion rod and obstetric chains placed on a fetus in a manner that will allow the obstetrician to correct the torsion by twisting the detorsion rod, applying sufficient pressure to

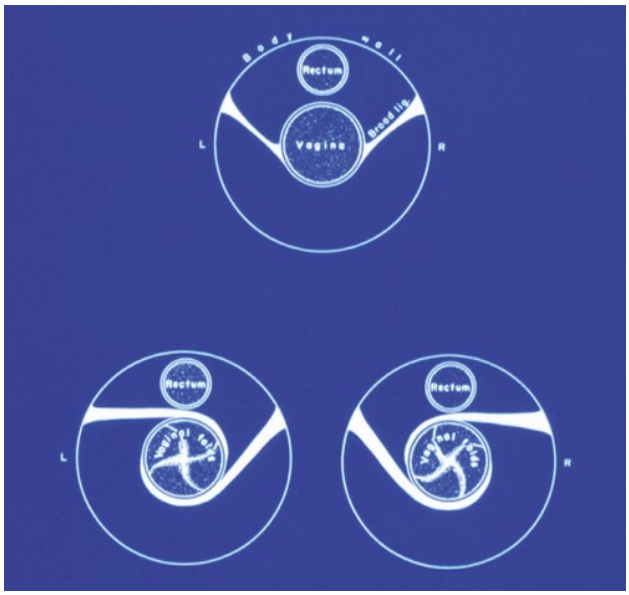


Figure 50.8 Relative positions of the broad ligaments of the uterus. Top image illustrates the normal relationship. Image lower left illustrates findings in a clockwise uterine torsion. Image lower right illustrates findings in a counterclockwise uterine torsion. *Source:* Image from [11], © 1971, Stephen J. Roberts.



Figure 50.9 Positioning of obstetrical chains and a detorsion rod for treatment of uterine torsion. The chains are placed as proximally as possible and threaded through the rings on each end of the instrument and secured to allow rotation of the fetus as the rod is twisted in the direction opposite the torsion. Careful monitoring to the limbs and chains during the rotation is necessary to prevent damage to the uterine mucosa.

rotate the fetus and uterus dorsally beyond the previously described point of balance. In skilled hands, utilization of a detorsion rod is often successful and requires less strength than the direct manual manipulation described previously. Proper placement of the chains can be difficult due to limited access related to the narrowing of the lumen of the vaginal vault and uterine body associated with the torsion.

Rolling a cow to correct uterine torsion is a commonly used technique and preferred over direct manipulation by many veterinarians. Following diagnosis and determination of the direction of torsion, the cow is cast into lateral recumbency on the side ipsilateral to the direction of the torsion. A long plank is placed over the caudal abdomen and a person of substantial size stands on the plank to immobilize the uterus as the cow is rotated through dorsal recumbency and into lateral recumbency on the opposite side. If the initial attempt is unsuccessful, the procedure may be repeated several times (Figure 50.10). The requirement for assistance limits the opportunity for application of this technique.

Following detorsion of the uterus, vaginal delivery of the fetus is possible if the cervix is sufficiently dilated to allow passage of the fetus. Cesarean section or fetotomy may be necessary should the cervix not dilate fully.

A standing flank laparotomy may be required to surgically alleviate the torsion when other methods fail. Vaginal delivery may be possible following surgical detorsion but hysterotomy is often required [14].

Hydropic Conditions

The accumulation of excessive uterine fluid in the pregnant uterus occurs sporadically in cattle as a manifestation of fetal fluid overproduction, failure of the placenta to appropriately remove fetal fluid, or a combination of those factors. Cases of pregnancy-associated uterine hydrops are divided into hydrops allantois when excessive accumulation of fluid occurs in the allantoic space, and the less common hydrops amnion in which an accumulation of excessive amnionic fluid occurs secondary to fetal defects. Hydropic conditions constitute an important component of the abnormal offspring syndrome sometimes associated with *in-vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT)-derived bovine pregnancies [16].

Hydrops allantois is the more common cause of uterine hydrops, accounting for greater than 90% of the cases of uterine dropsy. The pathophysiology leading to hydrops allantois has most often been attributed to defective placental function or placental insufficiency, and the placentae of affected fetuses frequently have a reduced number of placentomes with concurrent adventitious placentation. A cluster of hydrops allantois cases lacking placental abnormalities has been reported in closely related Japanese Black Cattle homozygous for a missense mutation in genetic coding for solute carrier family 12, member 1 (SLC12A1) protein. Fetal polyuria and excessive accumulation of allantoic fluid occurred due to the inability of the fetal kidney to reabsorb sodium. Genetic testing was developed and aggressively applied to identify carrier animals and reduce the occurrence of this simple autosomal recessive heritable condition in the breed [17].

Cases of hydrops allantois are uncommon events in most cattle populations, reportedly occurring in only one of 7500 pregnancies resulting from natural mating or artificial insemination. In one report, the incidence in pregnancies resulting from IVF or SCNT was significantly higher than in the wider cattle population, seen in as many as one in 200 such pregnancies as a component of abnormal offspring syndrome [18] related to altered gene expression and associated defective placental function from embryos matured and cultured in artificial media [16, 19].

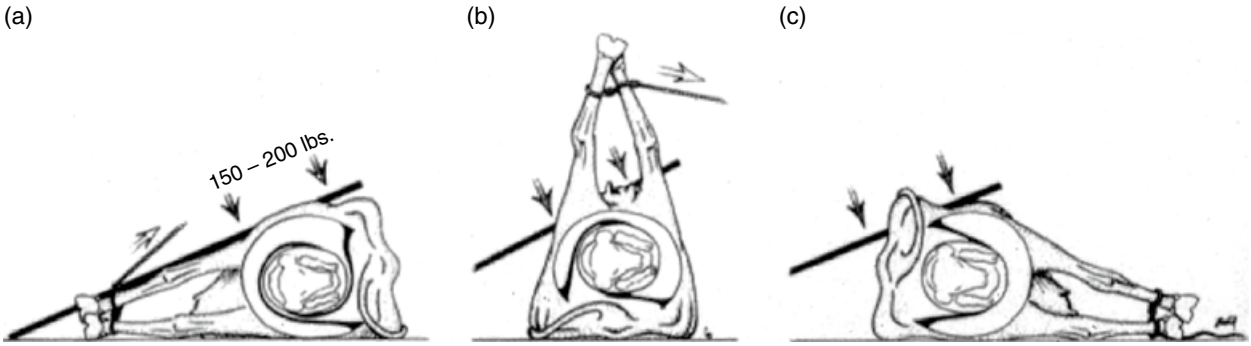


Figure 50.10 Schematic representation of the "plank in the flank" technique for correction of uterine torsion.

Hydrops allantois develops progressively after mid gestation, with the herdsman often reporting abdominal enlargement occurring over a few days, although the excessive accumulation of allantoic fluid likely began weeks prior to this being appreciated. Bilateral symmetrical abdominal distension is the most obvious finding, and external abdominal ballottement will elicit a fluid wave that crosses the abdomen (Figure 50.11). Cows are often thin by the time of diagnosis. The distended uterine wall can be palpated transrectally, but placentomes are difficult to discern and the fetus is usually not palpable. The fetus will seldom be viable following delivery and medical termination of the pregnancy or cesarean section is usually required to save the dam (Figure 50.12). Vaginal delivery will invariably require obstetrical assistance due to the lack of effective uterine contractions. Even with intensive medical management, the prognosis for life of the dam is guarded due to hypovolemic shock following fluid loss at the time of delivery and high risk of metritis post-partum. The prognosis for future fertility is poor. Humane euthanasia or salvage for slaughter is often indicated due to poor treatment outcomes.



Figure 50.11 Holstein cow with hydrops allantois. Note the bilateral abdominal distension and lack of mammary development. Ballottement of the abdomen induced an easily observable fluid wave.

Hydrops amnion is a rare gestational accident in cattle. The regulation of amnionic fluid volume is incompletely understood, but excessive accumulation can be seen in conjunction with craniofacial anomalies or neuromuscular disruptions which impair the ability of the fetus to swallow otherwise normal amnionic fluid. Excess amnionic fluid begins to accumulate slowly from mid-gestation to term [20], and the volume of amnionic fluid associated with amnionic hydrops can increase from the expected 2–3l [21] to 20l or more [22]. Abdominal enlargement resulting from excess accumulation of amnionic fluid may not be apparent until the last month to six weeks of gestation in affected pregnancies, and the excessive fluid volume may not be noticed until the time of parturition.

Cases of hydrops amnion have been related to congenital anomalies in which craniofacial defects in the fetus prevent the fetus from swallowing amnionic fluid [23, 24]. Heritable genetic defects, developmental disruption secondary to ingestion of teratogenic toxins, or viral infection during critical stages of fetal development can all initiate craniofacial fetal defects which may be accompanied by hydrops amnion. Pregnancy resulting from the breeding of American bison bulls to domestic cows frequently results in hydrops amnion [25].



Figure 50.12 Cow pictured in Figure 50.11 following a standing cesarean section.

Prepubic Tendon Rupture

The prepubic tendon is composed of the tendinous attachments of the rectus abdominis muscle, the internal and external abdominal oblique muscles, the pectineus muscle, and the gracilis muscles to the anterior border of the pubis [26]. This complex tendon provides a strong anchor point for the abdominal wall musculature to support the abdominal viscera. When rupture of the prepubic tendon or the closely related muscles of the ventral abdominal wall occurs, the loss of this anchor point is followed by progressive distension of the caudal ventral abdominal wall which may be accompanied by significant edema extending cranially to the umbilicus or beyond. The affected patient is painful and often becomes anorectic. Rupture of the prepubic tendon and associated caudal ventral abdominal musculature is seen almost exclusively in late gestation and may be associated with normal pregnancy, twin (or triplet, quadruplet, etc.) pregnancy, hydrops allantois [27], or prolonged gestation resulting in fetal giantism [11].

Prepubic tendon rupture carries a poor prognosis for the calf and for the life and future reproductive potential of the dam. Induction of parturition with assisted vaginal delivery or surgical delivery of the fetus(es) may preserve the life of the cow and sometimes result in delivery of a live calf provided the pregnancy is sufficiently advanced [11]. The inability of the dam to mount the abdominal press necessary for normal parturition mandates provision of obstetrical intervention at delivery.

Prolonged Gestation

Prolonged gestation occurs when spontaneous initiation of parturition does not occur at the expected time and duration of gestation far exceeds the upper limits of the normal range. Cases of pathologically prolonged gestation occurring as the result of defective development of the fetal hypothalamic–pituitary–adrenal axis associated with genetic mutations, teratogenic toxins, or infectious agents have been described.

Inherited autosomal recessive conditions in which dams pregnant with a fetus homozygous for a genetic mutation fail to go into labor have been described in both Holstein and Guernsey cattle [23]. As in the case with other autosomal recessive syndromes, the heterozygous parents (dam and sire) are unaffected and have a normal phenotype. The genetic anomaly producing excessive gestation length in Holsteins results in affected calves that are large at birth with fetal hirsutism, overgrown hooves, and small, nonfunctional adrenal glands [28]. A different heritable prolonged gestation syndrome described in Guernsey cattle resulting in agenesis of the fetal pituitary gland is characterized by small calves with severe craniofacial deformities [29]. Pregnancy duration in excess of 500 days has been reported.

Not all heritable causes of prolonged gestation are the result of these two well-described syndromes, and nonheritable developmental disrupters including toxins and viral infections which disrupt formation or function of the hypothalamic–pituitary–adrenal axis may interfere with the initiation of the normal parturition cascade.

Dystocia

Dystocia occurs when parturition is prolonged or difficult and results in increases in calf loss near the time of delivery and in increased incidence of morbidity and mortality of affected calves during the perinatal period. Dystocia may also be associated with decreased subsequent fertility in the dam due to delayed resumption of estrous cycles postpartum and decreased conception rates following insemination. Early recognition of the difficulty associated with the calving process and appropriate intervention can minimize but not eliminate herd-level economic impact.

Fetopelvic disproportion, in which the fetus is oversized relative to the maternal pelvis, is the most common cause of dystocia. Other common causes are related to inappropriate presentation, position, or posture of the fetus during delivery, simultaneous presentation of multiple offspring, metabolic disease (e.g. hypocalcemia) in the dam, and fetal anomalies. Dystocia is more common in primipara, with calving assistance required in up to 51.2% of first-calf heifers in one report from three Colorado dairy herds [30]. Heifer development programs designed to achieve adequate growth prior to breeding and during gestation in conjunction with sire selection for low to moderate birth weights are necessary components of dystocia reduction at first parturition.



Figure 50.13 Complete uterine prolapse following unassisted delivery. Hemorrhage from the uterine artery led to death in this case.

Uterine Prolapse

Uterine prolapse (Figure 50.13) sometimes occurs following parturition and constitutes a true emergency due to the prospect of life-threatening sequelae including hemorrhage and shock [31]. With early detection and prompt replacement, the prognosis for life of the patient is often good. Reports on the potential for rebreeding vary from moderate to good, and affected cows are not felt to be at increased risk for uterine prolapse at a subsequent calving [32–34]. Uterine lacerations, entrapment of the urinary bladder or intestine, severe desiccation of the exposed uterine tissues, and excessive blood loss leading to hypovolemic shock complicate case management and prognosis for return to function [34]. Amputation of the prolapsed uterus may be required for cases that are non-reducible or in which extensive uterine damage has occurred. More detailed descriptions of case management and reduction technique are found in Chapter 47 of this text and in several review articles [31–33].

Diagnosis is based on the appearance of the mucosal surface and the presence of caruncles on the everted uterus following parturition. The gravid horn may be prolapsed alone or portions of both uterine horns may be expelled. Predisposing factors include dystocia, with or without obstetrical assistance, hypocalcemia, and lateral or sternal recumbency with the forequarters elevated. Uterine prolapse represents a true veterinary emergency, and extreme care must be exercised while handling the patient to prevent damage to the exposed uterus and to minimize the possibility of rupture of the uterine arteries and subsequent hemorrhagic shock and exsanguination.

In most cases, the uterus can be cleansed and returned to the abdominal cavity after administration of epidural anesthesia to minimize straining as the prolapsed horns are reduced. The uterus should be examined carefully and lacerations repaired prior to reduction. On occasion, the bladder or other abdominal viscera may be contained within the prolapsed organ, complicating reduction. Following replacement of the uterus, care should be taken to fully extend the tips of the uterine horns to minimize recurrence. If concurrent hypocalcemia is present, administration of calcium borogluconate may be delayed until after reduction to minimize abdominal straining as the uterus is guided through the pelvis. Retention sutures, such as a Buhner stitch, should not be necessary if the tips of the uterine horns are appropriately positioned post reduction, but likely do little harm and may provide an additional level of security in cases in which pelvic trauma increases the likelihood of a continued abdominal press. Postoperative antibiotics and judicious use of ecbolic agents are indicated to promote uterine involution.

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51

Obstetrics: Mutation, Forced Extraction, and Fetotomy

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Introduction

The provision of obstetric services continues to be an important aspect of the large animal practice. Previous chapters have covered prevention (Chapter 49) and described and defined dystocia (Chapter 50). Chapter 52 covers the surgical management of dystocia (cesarean section). The purpose of this chapter is to describe the non-surgical obstetric management of dystocia as well as the decision parameters utilized to abandon one technique and proceed to another. This begins with a basic list of equipment and restraint options, and then progresses through the management of progressively more difficult obstetric cases.

Obstetric Terminology

Terminology that describes both normal and abnormal dystocia as well as techniques employed is valuable for effective case management discussion among both veterinarians and veterinary students. The following are accepted definitions of presentation, position, and posture which serve to describe fetal orientation with respect to the dam.

- *Presentation:* describes fetal orientation by the relationship of the spinal axis of the fetus to that of the dam: thus either longitudinal or transverse (sideways), and if longitudinal either cranial or caudal (cranial–longitudinal is normal), and if transverse either dorsal or ventral. A transverse presentation is very rare in cattle.
- *Position:* further describes fetal orientation based on the relationship of the dorsum of the fetus to the quadrants of the maternal pelvis, these being sacrum (dorsosacral is normal), right and left ilium, and pubis [1]. Note that while the dorsosacral position is considered to be the normal position for a fetus, slight rotation to a position midway between dorsosacral and right or left dorso-ilial will often facilitate delivery. In fact this is what typically occurs naturally as a cow shifts position from lateral to sternal recumbency during normal parturition.
- *Posture:* describes the relationship of extremities with respect to the body of the fetus. For example, for a forelimb this could be fetlock, carpal, or shoulder flexion.

Definitions for various obstetric techniques are as follow.

- *Mutation:* defined as those procedures by which a fetus is returned to its normal orientation with respect to presentation, position, and posture. Procedures utilized include repulsion (retropulsion), rotation, version, and extension of extremities [1, 2].
- *Forced extraction:* the manual or mechanically assisted removal of the calf through traction.
- *Fetotomy:* the reduction and removal of the fetus by division and removal of extremities and sections. In a percutaneous fetotomy, the dissection is made through the skin and this is the classic fetotomy procedure as described by Bierschwal and DeBois [3]. In a subcutaneous fetotomy, a "freeing" incision is made through the skin to allow a limb's bulk to be removed. This is typically a partial fetotomy in which only one or two extremities are removed.
- *Cesarean section*: the surgical removal of a fetus via laparohysterotomy.
- *Repulsion (retropulsion):* the pushing of the fetus back out of the pelvis to facilitate other corrective steps.
- *Rotation*: the turning of the fetus on its long axis.
- *Version*: the rotation of the fetus on its transverse axis.
- *Extension of extremities*: the correction of flexural abnormalities of the extremities.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

Basic Equipment

This is an area in which the opinions of obstetricians might vary. There are instruments that one individual might consider unnecessary that another would not approach an obstetric case without. Therefore the approach taken will be to first list obstetric tools that most would deem absolutely necessary and then add others and their potential uses [4].

Basic obstetric kit needs include at least two obstetric chains, two handles, a fetal extractor, a Krey hook, and basic surgery pack. Obstetric chains can be obtained in lengths of 53, 76, 114, and 152 cm. Chains are preferred over straps made for obstetrics for at least two reasons. The chains are more easily cleaned and despite the appearance are less likely to cause damage to the fetus or dam. With respect to handle selection, the old Muir obstetric handles are time tested but with the caveat that the heavier models are best. The type or model of fetal extractor is usually based on operator preference and its use is not controversial from my perspective. The Krey hook, which is routinely used in fetotomies, is listed among the basics as it is often useful in the vaginal delivery of a fetal abnormality or dead calf in which only a partial fetotomy is performed. A basic surgery pack is needed for such procedures as an episiotomy, a single appendage removal (partial fetotomy), or cesarean section (Figure 51.1). Additional obstetric tools that are useful include the calf-saver snare, which is easier to place around the head than using chains, a de-torsion bar, and a double blunt eye hook (Figure 51.2). Although limbs as well as the head and neck can be removed without a fetotome, to perform the classic fetotomy the following tools are necessary: a fetotome (model selected based on operator preference), saw wire, a wire guide, an "embryotomy" knife, and if not already in the obstetric kit a Krey hook (Figure 51.3).



Figure 51.1 Basic obstetric equipment: (A) 152-cm obstetric chain; (B) 76-cm obstetric chain; (C) Krey hook with attached chain; (D) T-bar obstetric chain handle; (E) Moore's obstetric handle; (F) components of disassembled fetal extractor.

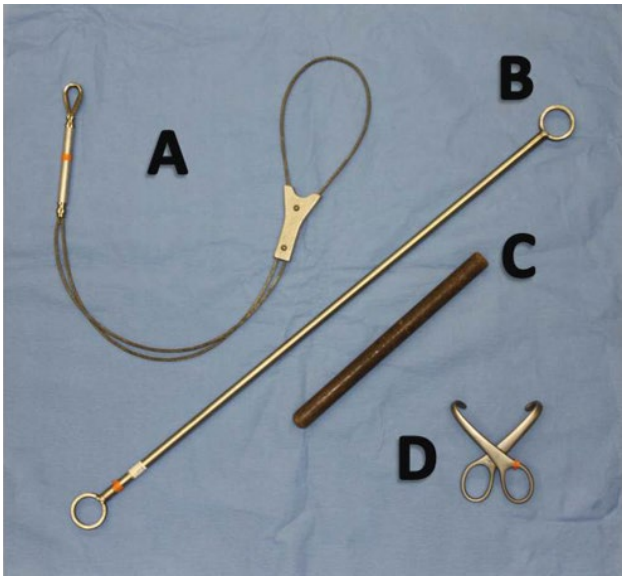


Figure 51.2 Additional obstetric equipment: (A) fetal head snare; (B) de-torsion bar or rod; (C) wooden dowel for applying torque to detorsion bar; (D) double blunt eye hooks or Vienna scissor eye hooks.



Figure 51.3 Fetotomy equipment: (A) Utrecht fetatome; (B) threader or insertion coil and wire brush; (C) Lyss wire handles; (D) wire handles; (E) Shriever wire introducer or passer; (F) Hauptner wire introducer or passer; (G) Linde's fetotomy palm knife; (H) Geunther's fetotomy finger knife; (I) side cutters to sever wire; (J) T-bar obstetric handle; (K) Krey hook with obstetric chain attached; (L) wire; (M) Moore's obstetric handle.

Case Management

Restraint

The restraint approach utilized must be safe for obstetrician and patient. Both facilities available and patient temperament dictate the approach taken. Haul-in facilities are almost always superior to those found on farms and indeed if a significant percentage of a practice is devoted to bovine obstetric work, suitable facilities are a good investment.

Although on-farm dystocia cases are often recumbent, most obstetric procedures and specifically mutation are easier on the well-restrained standing cow. Often a cow that has previously refused to stand will do so after the administration of an epidural, presumably due to the abatement from the pain associated with labor [1].

A cattle chute is adequate for examination, preparation, and the administration of an epidural, but you must have the ability to open a side or otherwise get the cow out in case she goes down during the obstetric procedure. In virtually all cases, with the exception of a purpose-designed dystocia chute or stanchion, you will be better off with the cow haltered and tied in the corner of a stall/pen/lot.

History

As the large-animal practitioner must often utilize to some extent a form of triage in determining which client's request or patient must be attended to first, the obstetric case begins with the perfunctory question of duration of labor. This may be of marginal value without an understanding on the part of the client of the stages of labor. For example, the time period that an owner relates is almost always from the time they first noticed the cow, without any respect to the stage of parturition. The stages of labor are as follows.

- Stage 1: early labor, with a typical duration of 4–12 hours. Its conclusion is marked by rupture of the allantois.
- Stage 2: delivery of the calf. This is also referred to as "true labor" and typically lasts 30 minutes to 4 hours, but the calf can often live 8–10 hours [4].
- Stage 3: passage of the fetal membranes.

Thus the appropriate question to ask the owner/attendant is whether or not fetal membranes or an extremity is visible, followed by queries to ascertain whether allantoic rupture has occurred ("Have the waters broken?"). Simply determining this provides the answer with respect to continued observation or immediate attention. Additionally, the knowledgeable stockman might be able to describe the presentation based on observation or their preliminary examination. Just as the knowledge that labor has progressed to stage 2 dictates prompt attention, so does a posterior presentation.

Other questions relate to parity, past calving history, and sire. The young veterinarian must realize that extensive questioning is often off-putting to the cattleman and one may be wise to simply expedite matters and obtain necessary information during the course of the management. The relative value of the cow versus the calf may also be important and will often be a factor in the management approach.

Examination

The examination begins as the cow is approached and as the animal is restrained. The temperament and physical condition are assessed. Prior to the obstetric examination, the tail should be tied to her neck (or held by an assistant) and the perineum cleaned with soap and water. The obstetric examination can be performed before or after the administration of an epidural, but at any rate should be done quickly and with the purpose of guiding the direction of obstetric management. Thus fetal viability, presentation, position, and posture, and degree of fetus–dam disproportion are evaluated.

Fetal viability and even how long a calf may have been dead are important determinants with respect to management. If the presentation and position are normal but an extremity is malpostured, the fetus can be repulsed and the posture corrected, followed by traction for example. A very large and viable calf in a heifer or relatively small cow directs one to cesarean section, as in turn a large dead fetus may dictate a fetotomy.

The presentation is easily assessed if the obstetrician can identify the head or conversely the tail/tailhead. When a determination must be made based on palpation of a limb, one should simply remember that the first two joints of the forelimb, the metacarpal joint (fetlock) and the carpus (knee) will both flex in the same direction, while the fetlock and hock flex in opposite directions.

The examination must therefore be performed in an efficient thoughtful manner with the goal of directing the first or next obstetric procedure and avoidance of the creation of a potential complication. It is intertwined with management, and reassessment of the case may result following an unexpected finding or the inability to complete a prescribed plan of action.

Obstetric Procedures and Decision-Making

As the reader would hopefully surmise from the section on examination, obstetric management is fluid, but should follow a reasonable course that results in a successful outcome, which in turn is dependent on the initial findings.

A common dystocia presentation is a fetus in anterior presentation with normal posture (for this and other dystocia images see Chapter 50). If traction results in a severe and immediate crossing of the forelimbs as the shoulders engage the maternal pelvis, the calf is likely too large to be delivered vaginally. Thus the next step would be to move to a cesarean section or fetotomy. In the case that the fetus is of a size in relation to the dam that vaginal delivery should be possible, examination to determine the cause of dystocia should follow. Was there adequate cervical dilation? Is the

heifer or cow exhausted or suffering uterine atony? Are there concurrent issues such as hypocalcemia? Is there inadequate dilation of the vulva? Depending on the answer, your next step could be to simply apply traction, but these issues should all be considered.

An anterior presentation in which the fetus is rotated in position based on head or limb orientation, findings from your vaginal examination, or findings of a rectal examination should lead to a diagnosis or suspicion of torsion. Diagnosis and obstetric management of uterine torsion is discussed in Chapter 50 and surgical management is discussed in Chapter 52.

For an anterior presentation with lateral deviation of the head, the fetus can be repulsed. This is obviously easier when the patient is standing and following epidural analgesia. The head can then be pulled into position (into the pelvis) by grasping the orbits. If the operator lacks the hand strength to do this, the double blunt eye hook can be employed. In the case in which they cannot be reached, first pull at the neck and this will pull the head closer. These attempts may be alternated with repulsion. Alternatively, or in cases when the head cannot be pulled into position, the calf-saver snare or an obstetric chain may be used. Correct application of the calf-saver snare and obstetric chains is important to avoid injury to the calf. They should be placed behind the ears and through the mouth. When utilizing a chain the loop should be placed in a manner so that it will not tighten when traction is employed. Also, traction can be utilized to move the head into position, but should not be used to effect delivery.

For an anterior presentation with one or more forelimbs back, the fetus is again repulsed and the limb(s) brought into position. Depending on the severity of the malpostured limb, correction may be stepwise, first reaching for and pulling on the upper aspect of the limb below the shoulder, then the carpus, and finally the fetlock. Affixing an obstetric chain around the fetlock and providing traction alternating with repulsion of the fetus is often necessary. Oftentimes only an elbow back is hindering delivery and is corrected by selective traction.

During the application of traction, once the thorax has cleared the pelvis, stop briefly and attend to the calf. Move any fetal membranes from over the calf's nose and mouth. Stimulate the calf if necessary by slapping the head or pulling on the tongue. Then before resuming traction, rotate the calf about 30–45°. This will likely prevent a "hip-lock" as can occur when the hips of the calf engage the maternal pelvis. In the situation when a hip-lock occurs the following tips are useful. If the cow is standing, orient the direction of traction almost straight down. If the cow is recumbent (lateral) at this time, first try the same change in orientation of traction. If a fetal extractor is employed, simply take the far end and direct it toward and between the cow's hind legs. Alternatively, or if the aforementioned technique does not work, rock the cow on her back, grabbing a hind foot, and roll her over (from right lateral recumbency to left or vice versa). Regardless, continued traction on a calf when there is a hip-lock without changing the direction of traction usually results in injury to calf and/or dam.

A posterior presentation in which the hindlimbs are flexed and back is the classical "breech." Correction requires repulsion of the fetus and, in a stepwise fashion, first flexing and retracting the hock, followed by the fetlock, and finally bringing each hind foot into position for delivery. This is extremely difficult in the recumbent animal, so all attempts to restrain the patient standing should be made and obviously, as whenever repulsion is necessary, epidural analgesia is employed. Care during repulsion is also crucial as uterine tearing can occur (etiology and repair is discussed in Chapter 55). If the cow or heifer has been in laborious efforts for an extended time, the reservoirs of fetal fluids for lubricating the genital tract will likely be exhausted and the uterus can be contracted to the point where performance of mutation(s) can be severely compromised. Pumping lubricant or water or a mixture of both through a nasogastric tube into the uterus can provide lubrication and separation of the uterus from the fetus, aiding mutation efforts. An additional tip to achieve uterine relaxation is to administer 10 ml of 1 : 1000 epinephrine intravenously to the cow/heifer [5, 6]. Be cautious utilizing this therapy, for there can be extreme consequences resulting in death due to side effects of epinephrine. This therapy should be considered on a case-by-case basis from the judgment of the clinician. This therapy will provide a tocolytic effect on the uterus that will allow mutational efforts of the fetus. Isoxsuprine and clenbuterol are also tocolytic agents that have been utilized in the bovine, but these two pharmaceuticals cannot be used in food-producing animals in the USA; therefore they are only mentioned here for reference. Once the malposture of both pelvic limbs is corrected, traction can be administered; however, with this, or in a normal posterior presentation, care must be taken to keep the calf's tail down during traction and delivery. Two possible deleterious sequelae can follow an upwardly fixed tail. First, due to the tension, the sharp ventral coccygeal processes can abrade or lacerate the dam's dorsal vagina. Second, and more commonly, the calf's tail will break, resulting in a neurologic deficit. With this injury the calf may still be able to walk, but lacks proprioception and additionally usually lacks bladder tone.

The goal for the management of dystocia is always to avoid injury first to the dam and then the calf. Prolonged obstetric efforts rarely end well so, regardless of the mutation techniques employed, a time limit of 10–15 minutes without

progress should lead to reevaluation of technique, and specifically in the case of a viable fetus to a cesarean section.

Forced Extraction

The term "forced extraction" refers to both manual and mechanically assisted extraction of the fetus. Since it is rare that problems (specifically injury) occur as a result of manual traction, the focus of this section is on traction in excess of that provided manually, thus referring specifically to the use of a fetal extractor. Because a fetal extractor can provide a level of traction that can be deleterious to either or both the dam and fetus, its use has been controversial. From a practical aspect its use is safe when used judicially.

Injuries that can result from forced extraction are significant and their consideration is important. Obturator or calving paralysis, which is more likely due to injury to or around the sciatic nerve rather than the obturator nerve, can occur as a result of prolonged dystocia, but is most commonly a result of excessive traction, as is tearing of the uterus, cervix, or vagina. Metacarpal and metatarsal fractures can result from incorrect placement of obstetric chains, but likewise are more likely with excessive traction.

This said, prevention of injury can virtually always be assured with adequate lubrication and careful application of traction. Methylcellulose-based lubricants can be pumped into the uterus and around the fetus, and then added during extraction. A note with respect to the selection of commercially available obstetric lubricants: the product J-Lube™ when exposed to the abdomen of cattle has resulted in death [7]. Thus if your obstetric management plan may potentially lead to a cesarean section and as abdominal contamination during this surgery is often unavoidable, its use is contraindicated. Traction should be applied slowly, carefully, and only in conjunction with the dam's contractions, and should be stopped when no progress results.

As stated previously, the model of fetal extractor utilized is based on operator preference. Remembering the old adage that no more traction should be applied than that of two strong men, the "power" requirement of a fetal extractor need not be excessive. Thus many of the time-tested older models are very adequate. A model developed several years ago features the ability to provide alternate traction to each of the two forelimbs. While it seems intuitive that this provides an advantage, in a well-constructed experimental model Becker et al. [8] concluded that simultaneous traction was optimal.

In summary, providing adequate lubrication, correctly applied obstetric chains, rotation of the fetus 30–45° during extraction, and applying traction with the dam's contraction efforts are all key to safe and judicious mechanical extraction.

Fetotomy

Fetotomy by definition is the dissection of the fetus into two or more parts while still within the uterus or vagina. Subcutaneous fetotomy was developed in the eighteenth century in which instruments were used to dismember a fetus while leaving the fetal skin intact to protect the uterus and provide points of traction [9]. This technique has been utilized far less since the development of the percutaneous fetotomy technique. The percutaneous fetotomy utilizes a braided wire as a saw to cut through skin, muscles, bone, and connective tissue to dissect or dismember the fetus into small portions for assisted vaginal delivery [3].

The standard Utrecht technique for a cranial or anterior presentation fetotomy is removal of the head, one forelimb, the other forelimb, and the trunk (potentially two cuts), and bisection of the fetal pelvis. This summarizes the complete fetotomy used to dismember an oversized fetus that has resulted in fetopelvic disproportion. This procedure will encompass five to six cuts to complete. A complete fetotomy is not always necessary for delivery of the fetus. Frequently a partial fetotomy, which may include one to two cuts, will provide the relief necessary to deliver the fetus. The standard cuts have been modified to incorporate more anatomical parts but which are still manageable portions easily removed from the genital tract [10].

Preparation for this procedure will depend on the environment in which the cow is confined and restrained, whether in a hospital, corral, or pasture setting. Deposition of obstetric lubricant or water mixed with lubricant can be infused into the uterus by the obstetrician based on their appraisal of its need to provide lubricating protection to the genital tract at any point during these obstetric procedures.

Anterior Presentation

If manipulative efforts to relieve the dystocia have not been successful and fetotomy is pursued, results of the manipulative process will determine the initial cut of the fetotomy. Malposture and malposition will have an impact on the initial cut as well. The fetotome should be threaded with a section of saw wire that is at least five times the length of the fetotome. This length of wire should be sufficient to allow a loop at the head of the fetotome that is large enough to be manipulated over the fetal head, as well as extension beyond the ends of both barrels of the fetotome at the handle to allow fixation of handles to the wire for sawing. This allows tension to be applied on the wire handles in an alternating manner to perform the cut. Tension applied to the wire handles will allow placement of the wire to be confirmed. The obstetrician should evaluate the wire saw as it exits the barrels of the fetotome to ensure that the wire is

not crossed just prior to entering each barrel. If so, this would lead to premature failure or breakage of the wire. The crossing of the saw wire can be alleviated by rotating the fetotome on its long axis. The appropriate direction is determined by the obstetrician after this evaluation. Initiation of the cutting action of the wire saw is conducted by exerting tension on the wire handles in an alternating manner. The amount of tension is increased. Initially the assistant makes short smooth strokes with the wire handles until the saw wire is fully engaged in the fetal tissue. The assistant can now extend the length of stroke and increase the amount of tension applied to the handles to perform the cut. The assistant or wire saw operator can determine when the cut is complete due to a dramatic decrease in resistance of the wire saw in tissue.

If the initial cut is intended to remove the fetal head, the threaded fetotome should be introduced into the genital tract. The loop of wire should be manipulated by hand to encompass the head. The head of the fetotome should be advanced into the tract so that it lies caudal to the ears. Tension may be placed on the wire after fetotome placement and the obstetrician can feel for placement of the wire around the neck. The standard Utrecht procedure describes placement of the fetotome head caudal to ramus of the mandible and potentially ventral as well. I prefer to advance the fetotome head to a location on the dorsum of the fetus at approximately the withers or craniomedial to both scapulae (Figures 51.4 and 51.5).

Apply tension to the wire saw handles and perform the cut through the fetal neck. The obstetrician can place a hand over the head of the fetotome during this cut to provide protection to the uterus dorsal to the instrument head. The obstetrician can provide traction to the fetal head for removal. It may be necessary to apply the fetal head snare or an obstetric chain if manual traction is unsuccessful at removal of the fetal part. This cut eliminates the neck as a potential impingement if traction is applied to the fetus at this point for delivery. This cut may also provide more medial excursion of the shoulders as they engage the maternal pelvis if traction is attempted at this time [11].

If removal of the fetal head does not provide satisfactory conditions for fetal extraction, the obstetrician can then proceed to removal of one of the thoracic limbs of the fetus. This can be accomplished by utilizing the previously threaded fetotome. A wire loop can be manipulated around the limb chosen for removal as the instrument is introduced into the genital tract. The instrument should be advanced along the lateral aspect of the limb to a point that the head of the instrument is dorsocaudal to the caudal extent of the scapula [1]. An obstetric chain may have been previously placed on the limb. Be careful not to entangle the chain in the saw wire. If a chain was not already in

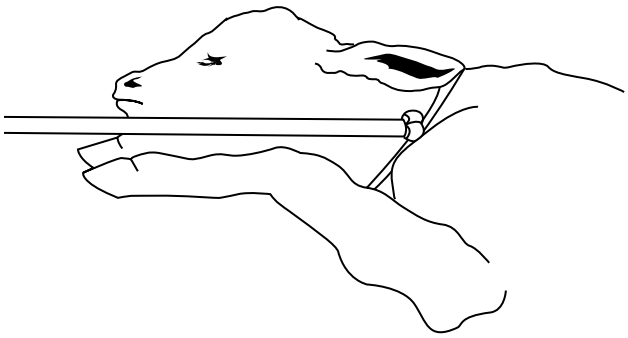


Figure 51.4 The author's recommended placement of a fetatome head for removal of fetal head and neck.

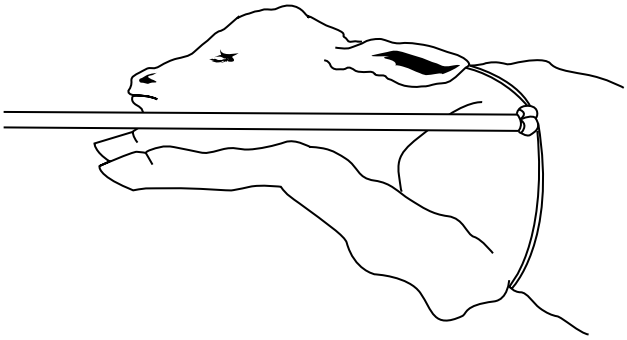


Figure 51.5 An alternate "cut" that incorporates the head and one shoulder, allowing removal of both with a single section.

place, one needs to be placed in order to provide traction during the cut. After instrument placement, tension on the wire will allow the obstetrician to determine if wire placement is correct. The wire should follow a path from the axillary region dorsally and ventrally to the upper limb and enter the instrument head dorsocaudal to the scapula. The obstetric chain can now have tension applied to it and the chain introduced into the locking plate at the handle of the fetotome. This will maintain the leg in full extension during the sawing action. The obstetrician may place a hand over the head of the fetotome during this cut to provide protection of the uterus. Once the cut is complete, the obstetrician can disengage the chain from the fetotome and apply traction to the fetal limb for removal. The instrument can be removed at this time as well. Palpation of the caudal region of the cut should be performed to ensure no bone fragments were left as a result of the cut that could potentially traumatize the genital tract. If fragments of the scapula are discovered, they can be removed by blunt dissection with the hand to prevent trauma to the genital tract if traction is attempted at this time [3].

Removal of the second thoracic limb is conducted in the same manner as the first. The same precautions should be taken to ensure that trauma to the genital tract from sharp bony fragments are minimized. Additional lubrication may

need to be deposited at this time due to multiple introductions and removals of equipment from the genital tract [12].

If an attempt is made at this time to extract the remaining portion of the fetus, evisceration of the thoracic cavity through the thoracic inlet can be conducted by hand. If the fetus is emphysematous, manual penetration through the diaphragm and evisceration of as much of the abdominal contents as possible manually through the thoracic inlet will accomplish a reduction in size of the remaining fetal portion. The Krey hook could be applied to the cranial end of the vertebral column as an appropriate place for tension application. To continue with the fetotomy, the head of the fetotome with loop of wire can be introduced into the genital tract, and the loop manipulated around the cranial end of the thoracic cavity. The loop is manipulated caudally on the trunk of the body to a point caudal to the last rib and cranial to the wings of the ilium in the lumbar region. The fetotome should be advanced into the genital tract so that the head rests in a dorsolateral position to the trunk of the fetus. It is sometimes difficult to manipulate the wire along the trunk of the fetus. The saw wire can be attached to a wire introducer or passer that is introduced into the genital tract. It is passed over the dorsal part of the trunk and then is retrieved by reaching under the ventral aspect of the fetal trunk. The wire introducer/passer is withdrawn from the genital tract. The saw wire is detached from the introducer/passer and threaded into the fetotome using a wire threader. The fetotome head should be positioned in the location previously described for this cut. The obstetrician should apply the Krey hook to the cranial end of the vertebral column. With the fetotome head in place, the obstetric chain attached to the Krey hook can have tension applied and engaged into the locking plate on the fetotome. The cut can be performed. If the thorax is of too large a diameter to pass through the genital tract, it can be reduced in size further by introducing the wire director/passer attached to the wire saw through the thoracic inlet and exiting the caudal opening created by the cut through the abdomen. The wire director/passer is retrieved from the caudal aspect of the trunk and withdrawn from the genital tract in order to thread the wire into the fetotome. The wire should be placed close to the attachment of the ribs to the thoracic vertebra. Performing the cut at this site will allow collapse of the thoracic cavity, which reduces its size for removal from the genital tract. Remain cognizant of sharp bony edges that can potentially traumatize the genital tract during manipulation. The assistant can provide traction to the chain while the obstetrician navigates the fetal thorax through the genital tract (Figure 51.6).

Positioning of the fetal pelvis for bisection can be accomplished by attaching the Krey hook to the lumbar vertebral column and applying traction to maintain the fetal portion

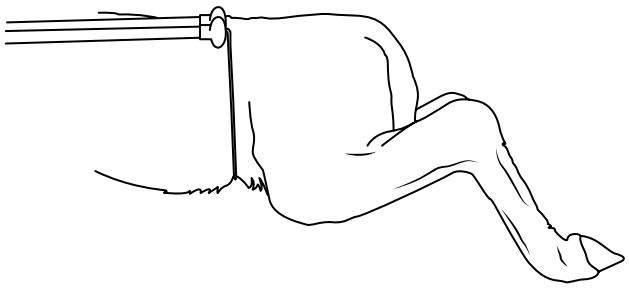


Figure 51.6 Wire placement for mid-abdomen cut.

in the opening of the maternal pelvic inlet. Saw wire from a half-threaded fetotome with wire introducer/passer attached can be introduced into the genital tract and passed over the dorsal aspect of the fetal pelvis to about the level of the inguinal region. The obstetrician can then retrieve the introducer by passing their hand between the cranial margin of the maternal pubis and fetal pelvis in order to grasp the introducer between the fetal limbs and withdraw it from the genital tract. The wire introducer can be removed and the wire saw can be threaded into the fetotome. The fetatome can then be introduced into the genital tract while tension is maintained on the wire saw to keep it in place. The head of the fetotome should be positioned so that it rests against the previously cut lumbar region of the fetus. It should be ipsilateral to the tail groove chosen for wire placement. It should also be slightly lateral to the vertebral column, so that this cut will bisect the fetal pelvis, but not in symmetrical pieces (Figure 51.7). With the fetatome in position, the chain attached to the Krey hook should have tension applied to it and then be engaged into the locking plate on the handle of the fetotome. The obstetrician can evaluate the placement of the wire saw; it should traverse a path from one barrel of the fetotome dorsally along the fetus, pass through the groove between the fetal tailhead and ischial tuberosity, and continue running ventrally through the inguinal region and along the ventrum of the fetus to enter into the opposite barrel of the fetotome [13]. If all is in order, the cut can be performed. The fetotome can be withdrawn from the genital tract when the cut is complete. The portion of the fetal pelvis that is grasped by the Krey hook can be guided out from the genital tract. The remaining final portion of the fetal pelvis can then be removed.

Posterior Presentation

If the calf was determined to be in posterior presentation during the genital examination, the following describes the procedures necessary to conduct a fetotomy in this presentation. Preparation of instrumentation and self will be the same as that for an anterior presentation. A fetotome threaded with the appropriate length wire saw and attached

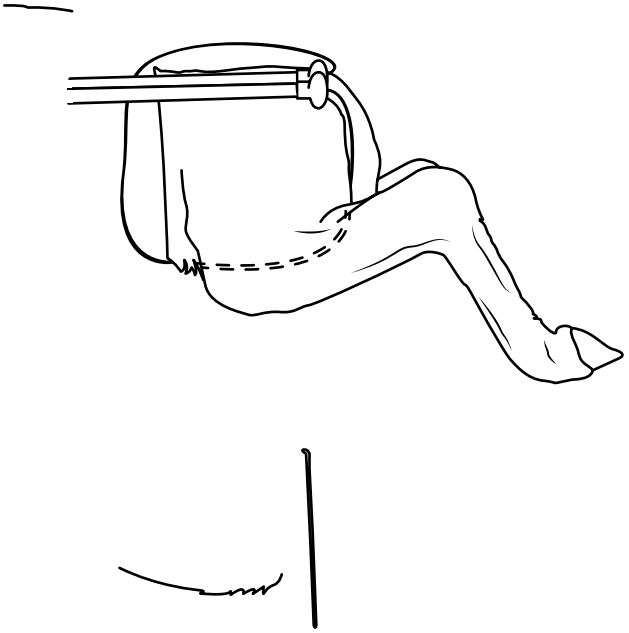


Figure 51.7 Wire placement for bisection of fetal pelvis.

wire saw handles should be available to initiate this procedure. The prepared fetotome can be introduced into the genital tract of the cow by the obstetrician. A loop of the wire saw protruding from the head of the fetotome can be manipulated over the claw of the selected pelvic limb to encircle it. The fetotome will be advanced similarly as for thoracic limb removal. It should be advanced along the lateral aspect of the limb so that the head of the fetotome comes to be positioned on the dorsal aspect of the pelvis in the area of the greater trochanter [1]. If a chain has not already been placed on the selected fetal limb, it should be placed at this time to provide traction during wire sawing. The chain should have tension applied to it and then be engaged into the locking plate on the fetotome handle. The cut can be performed to dissect the selected limb. When the cut is complete, the chain can be disengaged from the locking plate. The fetotome should be withdrawn, and the fetal pelvic limb can be removed from the genital tract.

A chain can be applied to the remaining pelvic limb, and traction applied to the chain to determine if the fetus can be delivered or additional dissection is necessary [3]. If the remaining fetal portion cannot be delivered, the obstetrician must determine whether the opposite pelvic limb requires amputation or dissection through the lumbar vertebra to allow for delivery of the remaining pelvic limb and pelvis. If the opposite pelvic limb requires amputation, it can be removed using the same technique as the first.

If the obstetrician chooses to dissect the fetus at the lumbar vertebra, there are two methods of wire placement. The obstetrician can choose to introduce a fully threaded fetotome into the genital tract and manipulate the wire loop around the claw to encircle it. The wire loop is manipulated proximally around the limb as the fetotome is advanced into the genital tract. The wire loop is manipulated cranially to a point that is just caudal to the last rib. The fetotome is advanced so that the head lies dorsolateral to the cranial lumbar vertebra. The second method uses a half-threaded fetotome. The wire saw exiting the barrel from the fetotome should have the wire introducer/passer attached. This instrument is introduced into the genital tract and advanced cranially to the region of the fetal lumbar area. The wire introducer is advanced ventrally on one side of the fetus and deposited. The obstetrician can retrieve the wire introducer by withdrawing the hand to the dorsal aspect of the fetus and then advancing it ventrally on the opposite side of the fetus to the area where the wire introducer was deposited. When the introducer is identified by the obstetrician's hand, it can be grasped and withdrawn from the genital tract so that the wire may be threaded into the fetotome. An obstetric chain should be applied to the pelvic limb if one has not already been placed. Once the fetotome is advanced into the proper position and wire placement is verified, traction must be applied to the obstetric chain, and it should be engaged into the locking plate of the fetotome handle with the limb in full extension. The cut can be performed at this time, and when the cut is complete the obstetric chain should be disengaged from the fetotome. The fetotome can be removed from the genital tract as well as the pelvis and attached limb.

Manual evisceration can be performed after the fetal pelvis has been removed which will reduce bulk in the abdomen of the fetus. A Krey hook and attached obstetric chain can be introduced into the genital tract and applied to the vertebral column so that controlled traction may be applied to retain the fetus in the maternal pelvic inlet while the fetotome is positioned for the next cut. To perform the next cut, a fully threaded fetotome can be introduced into the genital tract, and a loose wire saw loop should have the obstetric chain passed through it. The fetotome should be advanced cranially to the region of the caudal aspect of the scapulae, and the wire loop should be manipulated cranially to encircle the thorax so that it lies just caudal to the scapulae. Traction can be applied to the chain and it can then be engaged into the locking plate of the fetotome. Once fetotome and wire position are verified, the cut can be performed. The chain can be disengaged from the locking plate to allow removal of the fetotome. If this portion of the thorax is too large in diameter to be removed, it can be cut longitudinally along the rib attachment to the vertebral column as described for the anterior presentation.

After removal of the thorax, the remaining fetal forepart must be dissected. The Krey hook should be introduced to

the genital tract and applied to the fetus just lateral to the vertebral column. With moderate traction applied to the fetus, the obstetrician can proceed with the division of the fetal forepart of choice. This can be accomplished by either removing both forelimbs or diagonal longitudinal division of the forepart. I choose to perform diagonal longitudinal division of the forepart. To accomplish this, a half-threaded fetotome with wire introducer attached is utilized. The wire introducer is taken into the genital tract and is manipulated dorsally to the fetus and then ventrally between the neck and one forelimb to be deposited.

The obstetrician should withdraw the hand partially. To retrieve the wire introducer the hand should then be advanced ventral to the fetus to the area of deposition of the wire introducer. Once identified and grasped, the introducer should be withdrawn diagonally so that the wire comes to pass medially to the opposite elbow. The fetotome is threaded and advanced into the genital tract. The fetotome head should be positioned just lateral to the vertebral column on the opposite side from wire passage between the neck and forelimb. The Krey hook should be in position contralateral to the fetotome head and should not interfere with the wire saw. This cut will divide the forepart diagonally into unequal portions [3]. The fetotome can be removed after the cut is complete. Traction can be applied to the obstetric chain attached to the Krey hook to remove the fetal forepart it is grasping. The remaining fetal forepart should now be removed from the genital tract.

Subcutaneous Fetotomy

Subcutaneous fetotomy can be performed without special instrumentation. However, chisels have been fabricated and utilized in this procedure. Various scenarios may occur to which this procedure can be employed. Several modifications of this procedure have been described in the literature [14]. This procedure is utilized to remove a thoracic limb from a fetus, but it may also be employed for removal of a pelvic limb. The concept will be the same, but the exact method will vary tremendously due to major differences in anatomical structures between thoracic and pelvic limbs. The procedure will be briefly described as if removing a thoracic limb.

To perform this procedure, it is beneficial to have the fetal head cranial to the cervix to allow more access to the pelvic canal to perform the procedure. This can be accomplished by removal of the head or repulsing the fetus into the uterine body. Now that a thoracic limb has been chosen for the subcutaneous fetotomy, manipulate the limb into extension. A circumferential incision is made through the skin at the level of or proximal to the metacarpus (Figure 51.8).



Figure 51.8 Circumferential incision.

Proximal to the fetlock is the more suitable location because it potentially may be exterior to the vulva.

Second, an incision is made from the level of the scapulohumeral joint of the chosen limb descending down the cranial aspect of the limb to the recently made circumferential incision (Figure 51.9). The choice of incisional instrument is the preference of the veterinarian. Finger or palm fetotomy knives, scalpel blades, pocket knives [14], and utility knives are all cutting instruments that will accomplish the task. The utility knife does provide a mechanism for safety to the patient and operator due to function of the device by being able to project and retract the blade from the handle. The linear incision is more easily accomplished with tractional force applied to the limb. Alternatively, if one cannot easily begin the incision at the scapula, an incision can be made originating from the edge of the circumferential incision previously made (Figure 51.10). In either situation, the two corners of the incision formed by the linear and circumferential junction can be dissected and reflected caudally of the limb. Small incisions can be made in the area of these corners that can be utilized as finger holds while performing fetotomy, and later an OB chain can be placed in the hole to be utilized as a traction point.

Blunt dissection of the skin away from the limb should be performed to the level of the scapulohumeral joint. If one has a fetotomy chisel, it can now be employed to separate the skin from the limb (Figure 51.11). Digital dissection has been utilized to perform this procedure routinely

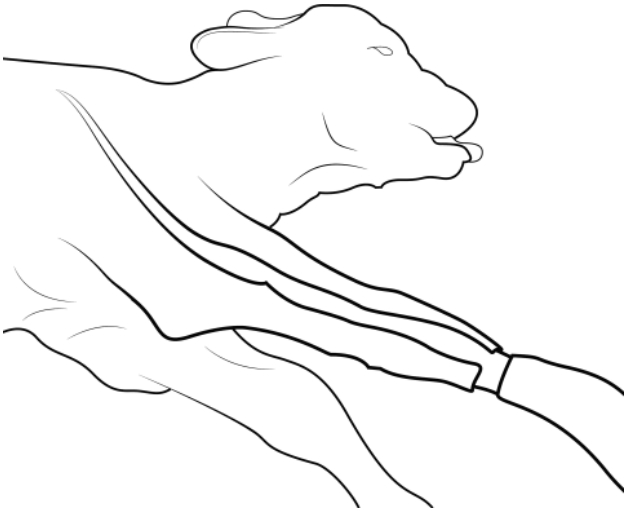


Figure 51.9 Incision started at the scapula of the fetus and extended toward the circumferential incision.

or a detorsion bar can be employed for skin separation in this procedure. Also, depending on the state of fetal decomposition, dissection of the skin from the musculature may not be necessary. When the skin dissection is completed to the level of the scapulohumeral joint, an OB chain can be applied to the distal limb and tractional forces applied to detach the thoracic limb from the fetus. Additional dissection will likely need to be employed if only manual force can be applied to the limb for detachment. Once the limb is detached from the fetus, it can be removed from the genital tract of the dam. Then proceed with further extraction efforts for removal of the entire fetus. The skin of the removed limb can be incorporated into points of attachment of OB instruments for removal of the remainder of the fetus from the dam.



Figure 51.10 Alternative incision originating from the edge of the circumferential incision.



Figure 51.11 Skin dissection with use of an obstetrical chisel.

Fetal Malpostures and Monstrosities

The obstetrician can be presented with a variety of fetal malpostures that result in dystocia. In each case, the obstetrician should use clinical judgment to determine if fetal manipulation and mutation are potentially hazardous to the genital tract of the dam. If this is determined to be true, then the obstetrician can approach the dystocia by performing custom fetotomy cuts to relieve the malpostures and allow for fetal delivery. The approaches to the cuts will be determined by the variety of malpostures.

Fetal monsters are another cause of dystocia where fetotomy should be given primary consideration. Because of variation in size, disfigurement, and flexibility of these fetal monsters, the obstetrician will have to determine the plan and approach for removal of such anomalies. The same principle of fetal size reduction should be applied to alleviate dystocia from such causes [3].

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52

Obstetrics: Cesarean Section

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Introduction

A cesarean section (C-section) is a surgical procedure that is utilized to relieve or in some cases prevent dystocia [1–3]. Causes of dystocia are categorized as maternal or fetal and are covered in Chapter 50. Indications for C-section include dystocia cases in which the calves are alive and valuable that cannot be delivered vaginally, and/or dystocia cases in which the calf is dead but delivery by another means jeopardizes the life or future fertility of the cow.

A C-section is rarely completed under strict asepsis, but this is not due to the fact that it is so often performed in field settings. This is even the case when a C-section is performed in a hospital setting in a surgery room. A C-section is classified as a clean contaminated procedure. This classification describes a surgery in which a non-sterile body cavity is entered under a controlled condition without unusual contamination; in this case, the uterus is the non-sterile cavity [4]. Furthermore, Mijten et al. demonstrated that uterine cultures collected during C-sections often resulted in positive bacterial cultures, but 83% of these samples were heavily contaminated, leading to the conclusion that endogenous bacterial contamination of the surgical site from the hysterotomy is likely inevitable [4]. The fact that bacterial contamination is probable does not excuse inattention to surgical preparation or to cleanliness. It is important to minimize exogenous bacterial contamination through efficient and quick performance of the procedure. Additionally, Mijten et al. recommended the administration of antimicrobials preoperatively to diminish postoperative complications as a result of endogenous contamination [4].

Success of a C-section is defined as a live cow with a live calf and the ability to rebreed the cow. These outcomes are dependent on a number of variables in addition to the surgeon's skill. Many of these variables are beyond the surgeon's control. A realistic approach to this surgery is to evaluate the case, the surgical theater, the cow's size, her condition and attitude, the owner's expectations, and the prognosis. Then tailor your plan to the circumstances and conditions presented. For example, there are several surgical approaches that have been described. It is typical for a surgeon to identify more with one approach than another due to preference and comfort level. However, there are specific case presentations for which a particular approach offers definite advantages. Newman discusses the importance of case selection when determining the surgical approach. To aid a surgeon's treatment plan, Newman categorizes the C-section procedure as elective, emergency non-emphysematous, and/or emergency emphysematous [5]. This categorization of the procedure is relative to the condition of the calf and its effects on prognosis and case management.

The elective category allows the surgeon to choose to some extent the time and the place that the surgery will be performed as well as the ability to make other preparations. Thus, this category carries the best prognosis for success, the exception being when the timing of the procedure might be elective but is performed due to an infirmity of the dam (fractured limb, etc.). The emergency obstetrical case in which a decision to proceed to a C-section is made early in the course of the dystocia without excessive manipulation and particularly while the calf is still alive likewise carries a very good prognosis. For both these case categories only basic postoperative management is required. The situation in which the decision to proceed to a C-section to deliver an emphysematous calf should carry with it the expectation or at least readiness for complications, significant postoperative care, and a guarded prognosis. Sometimes the decision to proceed to a C-section occurs after extensive obstetrical manipulation or even partial fetotomy efforts. This, in the opinion of the author, carries a less favorable prognosis than the emphysematous category.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Elective C-Section

Indications for an elective C-section are a valuable fetus, complicating factors such as a dam with a prepartum vaginal prolapse, a small or malformed pelvis, a recent life-threatening condition, and/or a prolonged gestation [1–3]. In any case, a history that includes breeding date or embryo transfer date should help assess the expected calving date. Calves born less than two weeks prematurely should not be unduly at risk for complications or fatalities (Chapter 48).

A C-section can be scheduled without respect to the initiation of parturition. Research performed comparing the timing of an elective C-section with respect to the stage of parturition revealed that the presence of full cervical dilatation before performing the C-section better allowed postnatal respiratory and metabolic adaptation in full-term calves [6]. When comparing an elective C-section to a vaginal delivery it is believed that aspects of vaginal delivery aid calves in transitioning from the womb. Specifically, in a study in which the hematological profiles of calves delivered by an elective C-section were compared to those of unassisted vaginal deliveries, C-section calves were uniformly more hypoxic and had a greater likelihood of experiencing respiratory distress [7]. Due to the experimental design, all of the deliveries (both vaginal and C-section) were uncomplicated. The hypoxia in the C-section calves was not due to or associated with respiratory or metabolic acidosis. Probo et al. hypothesized that during unassisted vaginal delivery calves benefitted from both the compression of the fetal thorax that occurs during transit through the pelvis and the temporary suspension by the hindlimbs improving postdelivery oxygenation [7]. Both of these events occur during spontaneous vaginal delivery.

To ameliorate these disadvantages, one option is to administer dexamethasone (20 mg) with or without a prostaglandin to induce parturition (Chapter 48). This of course makes the C-section less "elective." Certain techniques can be performed on the neonate post delivery to improve respiratory function. These are likely beneficial following the delivery of any calf. Carefully lifting the newborn by its rear legs as illustrated in Figure 52.1a for a few seconds should correspond with the stage of normal vaginal delivery in which the calf is momentarily suspended (Figure 52.1b). This must be performed without swinging or shaking the calf, which although a common practice can be injurious to the calf. Also note that lifting a newborn in this fashion could potentially result in excessive pressure to the diaphragm. Thus, closely follow the admonition that this be done quickly – specifically, less than 90 seconds. Following suspension, place the newborn in lateral recumbency. Grasp the thoracic limb, elevating and lowering the limb. This limb motion lifts the calf's chest off the ground, facilitating thoracic excursions. Repeat this motion three or four times. Additionally, or alternatively, respiratory efforts can be stimulated by "tickling" the nasal openings of the newborn with a straw or twig, or even an enteral feeding tube, inducing a sneeze, further aiding airway clearance (Figure 52.2).

Emergency C-Section

As stated, for prognostic reasons the decision to perform a C-section must occur in a timely manner. Several clinical signs or circumstances can direct a quick decision to proceed to surgery. The first clinical assessment indicating a

(a)



(b)



Figure 52.1 (a) Lifting a newborn calf by its rear limbs will duplicate aspects of natural vaginal delivery (b). *Do not shake or swing the calf. Source:* Image (b) courtesy of Colin Palmer.



Figure 52.2 "Tickling" the nasal openings of the newborn with an enteral feeding tube.

cesarean is the obviously small heifer with a small or an abnormally shaped pelvis [1–3]. Next, as obstetrical manipulations are made and extraction of the calf is being attempted, pressure on the proximal aspect of the fetal forelimbs by the pelvis resulting in crossing of the forelimbs is an indication of fetal–maternal pelvic size incompatibility. Additionally, failure to progress in delivery of the calf is an indication for surgery. Clinicians should have a preset metric (time frame) for progress during obstetrical procedures. For example, if obstetrical manipulation is being made without progress for a period of 10 minutes, then one must proceed to surgery. Note – this is not to say the calf must be delivered in that or another designated time frame, but that "progress" must be made.

In the case in which the fetus is dead and especially if it is emphysematous, the prognosis is less favorable. Case management and treatment plans must be discussed with the owner in regards to outcome prior to proceeding. Although a fetotomy is an option when the calf is dead, certain clinical presentations favor a C-section. C-section is the treatment of choice for the emphysematous breech, the unreducible uterine torsion, an undilated cervix, preexisting vaginal or cervical trauma from lay delivery attempts, and uncooperative patient temperament [1–3, 5]. These cases, as stated previously, dictate aggressive postoperative care and an increased risk of complications

Overview of Surgical Approaches

Surgeon preference, patient temperament, environment, and case presentation dictate the surgical approach selected. C-sections may be performed under standing sedation or cast in recumbency. The surgical approach determines the type of restraint employed and the method of anesthesia use, whether regional or general. Surgical approaches that have been utilized for C-section are the flank (left or right), the ventral midline, and the paramedian. There are no advantages of a paramedian over a ventral midline [8], so that approach will not be covered.

Standing Left Flank

The standing left flank approach is the most common surgical technique for C-section in cattle. The left flank approach is superior in that the rumen prevents prolapse of intestinal loops from the incision. This standing procedure is not recommended in animals that are likely to become recumbent during the surgery due to exhaustion, hypocalcemia, obturator nerve paralysis, and/or highly fractious nature [2].

Standing Right Flank

The standing right flank approach has limited indications. It can be utilized when there is ruminal distention or adhesions on the left side from a previous left flank surgery [9]. It has also been advocated as the best approach for a C-section performed due to hydrops allantois or hydrops amnii. In these cases, an attempt is made to remove the fetus and close the uterus without the massive fluid loss that will result in hypovolemic shock [8]. The right flank incision may also be utilized in the management of an uncorrected uterine torsion. The surgical technique for a right flank cesarean is similar to a left flank approach with the exception that on entering the abdomen extra care must be taken to avoid lacerating the small intestine, as the rumen is not available to serve as a shield [1–3]. It is recommended that the surgical incision does not extend to far ventrally, to prevent the small intestine from escaping the incision.

Ventral Midline Approach

The ventral midline is preferred by some clinicians as the primary approach utilized for C-sections. This approach has several advantages and there are some specific indications even for those that favor a standing flank method. The ventral midline approach is the preferred surgical technique for removal of an emphysematous fetus [1–3]. This technique allows for greater access to the uterus and minimizes abdominal contamination during delivery. The ventral midline also offers the benefits of closure of the linea alba, allowing for greater suture holding strength and less incisional hemorrhage when compared to the paramedian approach. The greatest disadvantage of the ventral midline technique is that it requires the cow to be cast in dorsal recumbency.

Cesarean for the Treatment of Uterine Torsion

A C-section must be performed to deliver the calf in cases when a uterine torsion cannot be corrected by other methods. Surgically assisted correction of the torsion via a flank laparotomy followed by vaginal delivery of the calf is preferred over a hysterotomy [8–10]. However, Frazer demonstrated that a third of all uterine torsion presenting to veterinary school referral hospitals resulted in C-sections [11]. Although either a standing flank laparotomy or a ventral midline approach can be utilized, the standing left flank approach will be described.

The incision for the flank laparotomy should be made about 25% longer than ordinarily done for a routine section. Once in the abdomen, an attempt is made to correct the torsion. A second operator utilizing a detorsion bar transvaginally may be utilized to assist this effort, but typically intra-abdominal manipulation is all that is necessary. Following reduction of the torsion, delivery of the calf is dependent on several factors. Fetal viability, uterine health, and cervical dilation dictate the manner of the delivery. If cervical dilation is sufficient, a vaginal delivery may be made at this time and the flank laparotomy closed. If dilation is not adequate or the calf viability is questionable, proceed with a hysterotomy.

In the situation in which the torsion cannot be corrected or the uterus ruptures during manipulation, realize that access to the hysterotomy and/or the uterine tear will become difficult as the uterus returns to its normal position. At this time, the author recommends a second approach from the right flank to assist in the closure of the hysterotomy and/or management of the tear. In both of these scenarios described, either the torsion that could not be corrected or the uterine rupture, the fetus is likely dead and the uterus has already undergone extensive damage. Thus, case management reflects heroic measures which carry a guarded prognosis. However, considering the value of some animals and the expectations of the modern owner, practicality is often dismissed.

Restraint and Anesthesia

Flank laparotomies can be performed with the patient standing or in right lateral recumbency. Restraint when performed standing can range from haltered and tethered to the almost complete immobility provided by a cattle "squeeze" chute. In fact, some cattle chutes with adjustable side panels can be set so narrow at the floor level as to prevent a cow from lying down.

An anesthetic technique described by Abrahamsen that provides analgesia and enhances patient cooperation while maintaining recumbency is the "Ketamine® stun" which is the administration of ketamine in combination with butorphanol and xylazine [11]. Specifically, it can be administered intramuscularly (IM) at a dose rate of butorphanol 0.01 mg/kg, xylazine 0.02 mg/kg, and ketamine 0.04 mg/kg. This will provide duration of effect for 45 minutes. Abrahamsen also reported a simplified protocol utilized originally by Dr. David Anderson while at Kansas State and simply referred to as the "5–10–20 technique." This refers to an approximate dose calculated for a 500-kg cow but reported to be safely utilized on cattle ranging from 340 to 660 kg. The combination dose is 5 mg butorphanol, 10 mg xylazine, and 20 mg ketamine administered IM to the patient [12]. The timing to onset of the clinical effect for this combination when administered prior to clipping and prepping the surgical site is typically perfect. Regional anesthesia options are the proximal paravertebral block or the inverted L block, both described in Chapter 17.

Surgical Procedure: Standing Flank Approach

The surgical site should be clipped and thoroughly scrubbed with chlorhexidine. The author recommends using cloth or paper drapes to cover the chute to reduce contamination of the exposed uterus (Figure 52.3). A vertical skin incision is made in the middle of the paralumbar fossa, beginning 10 cm ventral to the transverse vertebral processes and extending distally for a distance of 25 cm [1–3]. The incision is extended to a length sufficient to allow extraction of the calf. Alternatively, an oblique celiotomy incision can be made [13]. This skin incision is begun 8–10 cm cranial and 8–10 cm ventral to the cranial aspect of the tuber coxae and extended cranioventrally at a 45° angle, ending 3 cm caudal to the last rib. The oblique approach allows for better access to the gravid uterus.



Figure 52.3 Surgical pack.

Incision of the skin and the subcutaneous tissue exposes the fibers of the external abdominal oblique muscle. This muscle is incised vertically the entire length of the skin incision. Next the internal abdominal oblique muscle is incised in a similar fashion. Upon completion of this incision the aponeurosis of the transverse abdominal muscle is revealed. Care must be taken in this step to prevent inadvertent entrances into the rumen. To avoid perforating the rumen, tent the transverse abdominal muscle with tissue forceps to make a small incision through the muscle and the peritoneum with a scalpel blade or Metzenbaum scissors. Once a small opening is made, elongate the incision using the tissue forceps as a tissue guard. Incise the remaining transverse abdominal muscle and the peritoneum with the blade or the Metzenbaum scissors. When the peritoneum is incised and the abdominal cavity opened the sound of air entering the potential space is heard. The rumen is identified and pushed cranially to allow access to the uterus (Figure 52.4). In the case of a fetus in a normal anterior position, a hindlimb within the gravid horn will be both the most recognizable and most accessible part of the calf. The distal hindlimb is used as a handle to exteriorize the uterus through the abdominal incision (Figure 52.5).



Figure 52.4 The rumen is identified and pushed cranially to allow access to the uterus.

When the part of the uterus that encases the distal limb is exteriorized the hock is then identified and "hooked" outside the incision. The uterus is incised along the greater curvature, and care must be taken to incise only the gravid horn and avoid the uterine body. The incision begins over the hock and extends to the claw. Care must be taken to avoid incising the placentomes and the fetus itself. Be forewarned that incisions that are too short will result in tearing of the uterine wall as the calf is delivered. These tears are always ragged and somewhat perpendicular to the incision line, complicating subsequent closure. After the incision is made, obstetrical chains can be attached to facilitate delivery. Following delivery of the calf the uterus is then examined: for the presence of another fetus, uterine trauma, and tears of the uterine wall.

Uterine Closure

The placenta is not removed unless it is easily detached. Care must be taken not to include the placenta in the uterine closure. Often it is helpful to trim away some of the



Figure 52.5 In the case of a fetus in a normal anterior position, a hind limb within the gravid horn will be both the most recognizable and most accessible part of the calf. It can then be grasped and used as a handle to exteriorize the uterus through the abdominal incision.

membranes from the incision prior to closure to prevent incorporating it into the suture line. The uterus is then closed with an inverting continuous suture pattern. The Cushing pattern, a modified Cushing pattern, or the Utrecht uterine suture is recommended (Figures 52.6–52.10). The advantages of the Utrecht pattern are reduced postoperative adhesions because the suture and knots are buried [3]. A tapered needle and chromic or plain catgut suture (no. 2 or 3) is recommended as at least one study comparing plain catgut to polyglactin 910 revealed no significant advantage to the use of the synthetic product [14]. A single-layer closure, utilizing the Utrecht pattern, is adequate unless there is concern with regard to the competency of the first suture line or the condition of the uterus. In cases in which the uterus contains severely contaminated fluid, a two-layer closure is indicated. A simple continuous suture pattern followed by a Cushing pattern improves the competency of the closure in a contaminated environment. Regardless of the suture pattern chosen for closure, it is important not to delay suturing as the uterus begins to involute quickly [3].

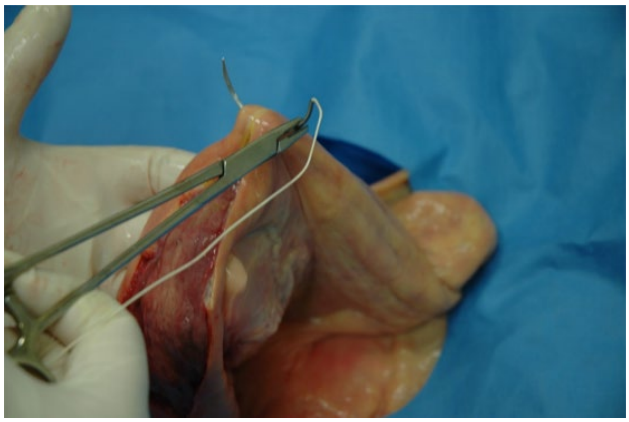


Figure 52.6 Initiating the Utrecht suture pattern, begin the suture line proximal to the uterine incision; do not enter the lumen of the uterus.

Once closure of the uterus is complete, removal of blood clots and contamination from the serosal surface is imperative in reducing postoperative adhesions (Figure 52.11). Following closure of the uterus the administration of 10–20 units of oxytocin into the uterine wall and 30–40 units intravenous (IV) will hasten uterine involution and aid in passage of the placenta (Figure 52.12).

Then, depending on the level of abdominal contamination, it may be advisable to lavage the abdomen with large volumes of saline to remove clotted blood and debris. If there is gross, severe contamination, large volumes can be used and egress of lavage fluids can be facilitated by placement of a rumen trocar through the ventral abdomen, as described in Chapter 55. In addition, carboxymethylcellulose, heparin, potassium penicillin, flunixin meglumine, glycerol, and tetracycline have all been advocated as components of a postsurgical abdominal lavage [5, 15–17].

When the exteriorized portion of the uterus has been maintained outside the abdomen for delivery and uterine closure, it can be assumed that there is no abdominal contamination. Contamination must be assumed in cases such as an unresolved uterine torsion, a fetus in the "breech" posterior presentation, or large fetal size preventing the complete exteriorization of the uterus [1]. This raises a couple of issues. First, a cautionary note with respect to the use of a popular obstetrical lubricant, J-Lube™, which has been shown to be fatal to cattle, horses, and mice following intra-abdominal exposure [16]. Thus, because abdominal contamination by the uterine contents is always a possibility, it is recommended to avoid the use of J-Lube in obstetrical cases that

(a) (b)

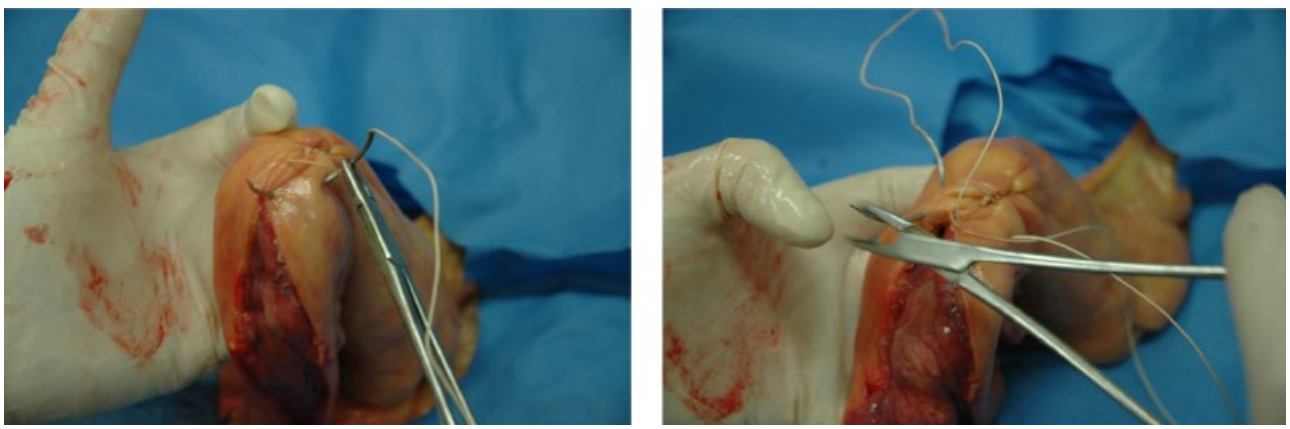


Figure 52.7 (a) and (b) Once the knot is secure, back place each bite at a 45° angle oblique to the incision proximal to the exit of the previous bite.



Figure 52.8 Tighten the incision by pushing the uterus away from the suture line. This gives the closure a bunched or pursed appearance.



Figure 52.9 Completion of the Utrecht suture line to bury the knot.

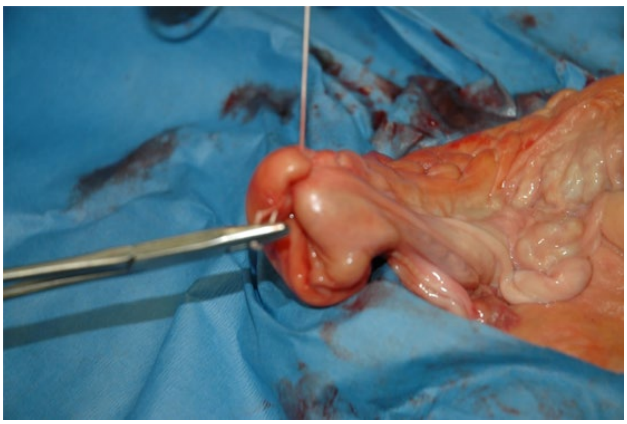


Figure 52.10 Note that as the Utrecht knot is tightened, the remaining suture is covered.

ultimately result in a C-section. Conversely, sodium carboxymethylcellulose, which is a very good obstetrical lubricant, not only is safe but also has been used in abdominal lavage fluids to minimize adhesions [16].

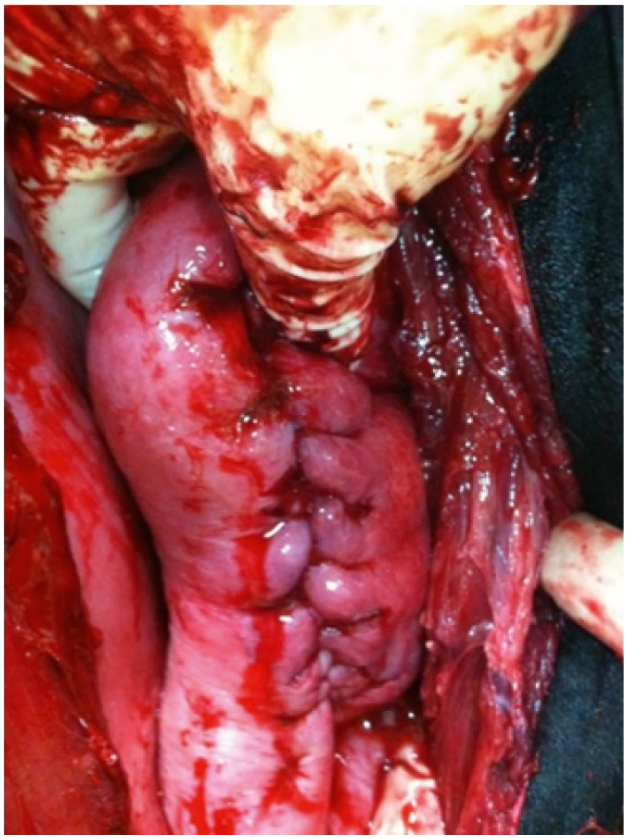


Figure 52.11 This image demonstrates the accumulation of blood clots along the incision that incite the formation of adhesions, reducing fertility. Notice how the uterus is withdrawn into the incision. A surgeon must work quickly due to the rapid involution of the uterus.

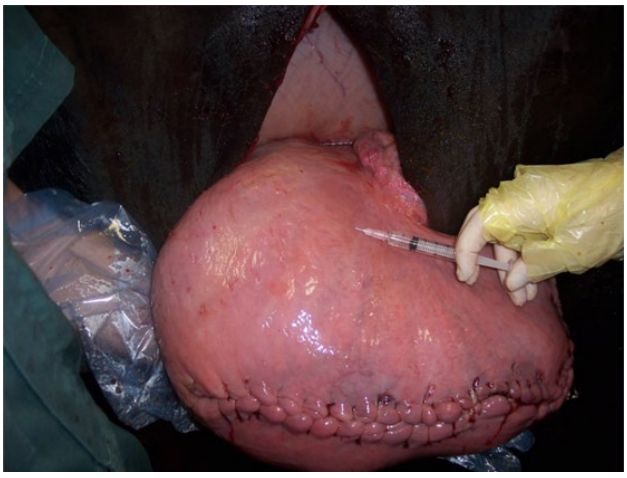


Figure 52.12 Injection of oxytocin into the uterine wall prior to abdominal closure.

Body Wall Closure

Abdominal wall closure is achieved in three layers. The peritoneum and the transverse abdominal muscle are closed together with simple continuous suture pattern using no 2. or

3 chromic catgut or synthetic no. 3 polyglactin 910. The internal and external abdominal oblique muscles as well as the subcutaneous fascia are closed with a simple continuous suture pattern using no. 3 polyglactin 910. The author recommends closure beginning distally and working proximally to help contain viscera within the abdominal cavity. Skin closure can be performed with 0.6-mm nylon (Braunamid™) utilizing a pattern of the surgeon's choice [1–3].

Surgical Procedure: Ventral Midline Approach

The cow is cast in dorsal recumbency with both the head and the forelimbs tied in extension (Figure 52.13). The abdomen is clipped and aseptically prepped. Regional anesthesia is performed with a line block utilizing 2% lidocaine, being sure to inject within the subcutaneous and intramuscular spaces (Figure 52.14). A midline incision is initiated immediately cranial to the udder extending cranially 5–7 cm beyond the umbilicus [2]. Once the skin and subcutaneous tissues are incised, a small stab incision is made through the linea alba. This stab incision is large enough to pass a finger or forceps to elevate the body wall to allow extension of the incision along the linea while minimizing risk of abdominal visceral penetration. Once the abdomen is entered, downward pressure on the body wall will help to push the gravid horn of the uterus toward the incision. To minimize gross contamination to the abdomen, a sterilely packaged plastic Mayo stand cover works well to isolate the uterus. Make a small incision into the Mayo cover, pulling the gravid horn through the Mayo stand cover. Tear the opening so that the cover is tightly adhered to the uterus but allows enough room for manipulation. Place the Mayo cover so that the abdominal incision and viscera are covered and only the gravid horn is exposed (Figure 52.15). Manipulation of the uterus, delivery of the calf, and closure of the uterus are as previously described.



Figure 52.13 Cow cast in dorsal recumbency with both head and forelimbs tied in extension.



Figure 52.14 Regional anesthesia supplied by a line block utilizing 2% lidocaine injection within the subcutaneous and intramuscular spaces.

Closure of the linea alba and peritoneum is obtained by using either a simple continuous or near-far-far-near pattern with no. 3 polyglactin 910. To facilitate closure, placement of towel clamps along the incision or several interrupted cruciate sutures will help alleviate tension as the incision is closed. The subcutaneous tissues are apposed with absorbable suture in a simple continuous suture pattern. The skin should be closed with 0.6-mm nylon (Braunamid) in a simple interrupted cruciate pattern; continuous patterns are difficult to remove from a ventral midline incision.

Postoperative Management and Complications

Complications following C-section include endotoxemia, peritonitis, metritis, adhesions, and reduced fertility [1–3, 5]. Mostly these complications are the result of bacterial contamination. As previously stated, all cesareans should be considered clean-contaminated surgeries even in the best of circumstances. Therefore broad-spectrum antibiotics and non-steroidal anti-inflammatories should be administered prior to surgery. Systemic antibiotics should be maintained for three to five days following surgery depending on the degree of surgical contamination. Severely endotoxic animals by definition should be treated with antimicrobials several days beyond the resolution of



Figure 52.15 (a) and (b) The gravid horn has been placed like a "sack" inside a sterile disposable Mayo stand cover, then the remaining Mayo cover is packed within the incision, protecting the abdominal viscera.

the endotoxemia. Endotoxic animals will require additional cardiovascular support of IV fluid therapy.

Clinical signs of peritonitis, pyrexia, inappetence, and ileus usually become apparent by three days following surgery. In the absence of these clinical signs, antibiotics may be discontinued.

Intra-abdominal adhesions can reduce future fertility. The chapter has previously stated that utilizing the Utrecht suture pattern and removing blood clots from the uterine serosa will remarkably reduce postoperative adhesions. The author recommends using 11 of carboxymethylcellulose prior to body wall closure to further decrease the risk of postoperative adhesions.

Postoperative fertility is reduced by approximately 15% of that of cows that did not have a C-section, with postoperative fertility rates expected to be between 60 and 80% [1, 2].

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53

Retained Fetal Membranes

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Introduction

Placenta is the transporting epithelium between fetal and maternal circulations; it is created and owned by the fetus, protected by the dam, escapes antigenic rejection, loses its purpose immediately after rupture of the umbilical cord, and is expelled after fetal delivery. Bovine placenta fascinated early farmers, excited many scholars to create more than 3000 documents since 1919, including the author [1], and continues to provide remuneration for veterinarians all over the globe because of its retention. Failure of placental expulsion constitutes retained placenta. Much has been documented regarding retained placenta in theriogenology textbooks [2–6] based on these authors' own vast clinical experience and information gleaned from scientific publications. Excellent review articles by leading veterinary clinical investigators have more vividly encapsulated the various aspects of this clinical condition [7–25]. What is known about its clinical signs and negative influence on postpartum reproductive performance has not changed in the last century. However, over the years, many hypotheses with regard to its occurrence (particularly at the tissue and cellular levels), risk factors, preventive measures, and treatment plans have been proposed. Headings for this topic include definitions, developmental morphology of placenta to better understand the process of detachment and etiology, pathogenesis, treatment, and prevention. It will become very apparent on reading this chapter that there are many instructive similarities among these topics.

Definitions

The fetal part of the placenta, namely the fetal membrane (allantochorion), normally separates from its maternal moorings during the process of parturition. The process of parturition is marked by vascular, contractile, and mechanical dynamics designed to expel the fetus and its membranes (allantochorion and amnion). Fetal and maternal parts of the placenta have equal regulatory roles in the timely separation of the fetal part of placenta. Hence, use of the term "placenta" in this chapter will point to a collective structure created by dam and fetus. Retention is primarily due to failure of the villi of the fetal cotyledons to detach themselves from the maternal crypts of the caruncle. The process of separation and inversion of the allantochorion (allantoic surface faces outside) is completed on average within eight hours of initiation of parturition, depending on the parity and age of patient. In general, all parts of the allantochorion and amnion need to be expelled within 0.5–12 hours after calving. Thus retention of fetal membranes is referred to as retained placenta or, more correctly, as retained fetal membranes (RFM), with the term "fetal membranes" referring to both the allantochorion and amnion. While the use of "fetal membrane" (singular) for allantochorion is accepted, to avoid confusion the term "allantochorion" will be used in this chapter.

Developmental Morphology

Presently, bovine placenta is described as cotyledonary synepitheliochorial based on the morphology that is established around 40–50 days into pregnancy. Earlier classification as syndesmochorial was based on a misunderstanding of the number and form of the layers intervening between fetal and maternal circulation. This classification needed correction because of new evidence. The earlier assumption that the uterine epithelium is lost in the process of placentation resulting in direct apposition of trophectoderm to the maternal connective tissue is no longer tenable. Now it is known that uterine epithelium persists, although initially modified

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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to a variable degree into patches of a hybrid fetomaternal syncytium formed by migration and fusion of a particular type of trophectodermal cells with uterine epithelial (UE) cells. These trophoblast binucleate/giant cells (TGC) migrate and modify uterine epithelium by apical fusion to form fetomaternal hybrid syncytial plaques with up to eight nuclei at the junction of fetal and maternal tissue. These syncytial plaques are replaced by regrowth of UE cells by day 40, and subsequently TGC–UE fusion produces only transient trinucleate minisyncytia throughout the remainder of pregnancy.

Research findings have necessitated a change in the morphological terminology of placentation and defined bovine placenta as synepitheliochorial. The prefix "syn" indicates the contribution of TGC to fetomaternal syncytium and contrasts with the simple microvillar interdigitation between trophoblast and uterine epithelium (epitheliochorial) over the rest of the placenta. Cotyledonary refers to the presence of localized areas of trophectodermal proliferation forming "cotyledons" in placenta and each cotyledon is the fetal part of a placentome. Placentome is formed by the tuft of branching chorionic villi from the cotyledon that grow and enmesh with corresponding maternal caruncular crypts, providing a finger-in-glove arrangement. These crypts develop from the preformed flat endometrial caruncles (Figure 53.1), present in the non-pregnant cow, which are aligned along the uterine horns. Normally, placenta consists of 80–90 of these placentomes. The placentomes are linked together by areas of flat apposition between trophoblast and caruncular epithelium (CE). The CE cells are similar to UE cells and are homogeneous in population unlike trophoblast. Placentomes guarantee a firm anchorage by the complementary interdigitation of fetal villous trees with maternal crypts and by the interdigitation of the apical microvilli from uninucleate trophoblast cells (UTC) and CE directly by cell–cell contact or indirectly by cell–matrix contact. Secondly, placentome formation with a synepitheliochorial interhemal barrier provides vast increase in surface area necessary for the continuously increasing substance exchange between dam and fetus. It is important to note that placentomal gross morphology and the pattern of fetomaternal interdigitation (villus/crypt architecture of the mid to late pregnant placenta) differ considerably between bovid species but detailed cellular structure of the maternofetal interface is similar throughout pregnancy. It is pertinent to point out that no maternal tissue is shed in the afterbirth (secundines) of cattle. On the other hand, a few fetal villi may be caught and left in maternal crypts.

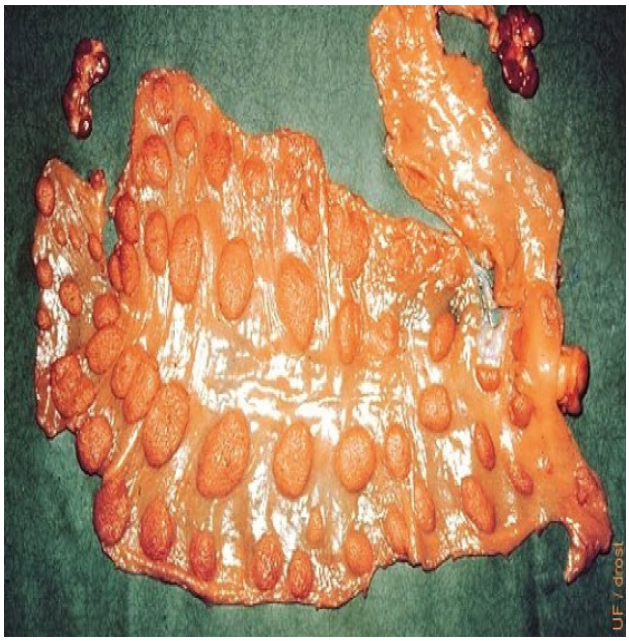


Figure 53.1 Caruncles are arranged in four rows with each row approximately 15 inches long. *Source:* Courtesy of Maarten Drost.

Etiology

Placentomal maturation coupled with appropriate structural, endocrinological, and immunological changes result in easy separation and expulsion of the fetal membranes. Furthermore, vascular, contractile, and involution changes that occur in the uterus before and during parturition help in the separation and expulsion of fetal membranes. It should be very well recognized that the process of maturation and timely separation and expulsion of fetal membranes involves well-orchestrated regulation by both maternal and fetal components of the placenta.

Placentome Maturation

It has been established that a physiological parturition occurs on the face of mature placental structures. The fact that induction of parturition is followed by RFM in most cases supports the observation that immaturity of placentome may be one of the contributing factors for RFM since parturition is initiated before term in such cases. Similarly, that RFM has been an issue in most abortion cases lends credence to the notion that a mature placentome is a prerequisite for the separation of allantochorion.

Endocrine Changes

Maturation of placenta is followed by specific endocrine changes. These changes are important to bring about contractile and apoptotic events, and expression of genes for

matrix metalloproteinases and their inhibitors. Although a mature placenta is capable of initiating favorable steroidogenic and arachidonic cascades, altered expression of antioxidative defense mechanisms at this juncture can interfere with steroidogenesis at the cellular metabolic level. Poor expression of the antioxidative defense mechanisms against reactive oxygen species (ROS) can result in an imbalance between production and neutralization of ROS, preventing proper detachment of the allantochorion.

Closer to parturition, increases in peripheral estradiol concentrations and less perceptible changes in progesterone are observed. Progesterone perfuses into placentomes which may contribute to its imbibition within placentome, enabling structural, and functional changes. These changes facilitate contraction of myometrium by estradiol along with other hormones including oxytocin and specific prostaglandins. It is important to note that these structural changes can possibly occur only in a mature placentome that has been exposed to adequate duration and quantity of estradiol.

Regarding prostaglandins, the type, and quantity of prostaglandins produced can influence the contractility of the myometrium. For example, a lower ratio of prostaglandin (PG)E_2 to $PGF_{2\alpha}$ within fetomaternal compartments of placentome and an elevated steroid hormone receptor status possibly reduces the rate of apoptosis occurring in chorionic epithelium before calving. There may be other unknown factors involved in the regulation of apoptosis; nevertheless, it is recognized that dysfunctional expression of apoptotic regulating factors can contribute to failure of separation.

Expression of matrix metalloproteinases and counteracting tissue inhibitors of metalloproteinases in the fetal compartment are essential to balance extracellular matrix formation and degradation, critical in the separation of the allantochorion. Specifically, they are responsible in breaking down collagen within the villi and assisting in the separation of villi from the crypts.

Finally, it should be remembered that despite the complexity of various prostaglandins and their production and our incomplete understanding of their specific roles, the use of prostaglandins to prevent the occurrence of RFM in induced parturition has yielded inconclusive results.

Structural Changes

Although the tight connection within placentome is essential during pregnancy, it has to cease to allow allantochorion separation. Indeed, distinct remodeling and loosening of adherence occurs within placentome during late pregnancy. Transformations that occur in placental epithelium and connective tissue during the last month of pregnancy prepare the placentome for critical loosening of tissues involved in fetomaternal interdigitation. This process has to proceed undisturbed and has to culminate in separation of allantochorion at the right time. Changes include progressive collagenization of maternal and fetal connective tissues, flattening of maternal epithelium lining crypts nearest to the caruncular stalk, and appearance of TGC that become polynuclear shortly before the detachment process. Currently, research is focused on studying the difference in protein profile of retained and non-retained fetal membranes, which may shed further light on etiology [26].

Immunological Changes

Expression of major histocompatibility complex (MHC) class I in bovine trophoblast cells and their tight regulation are biologically relevant. Early in pregnancy, a complete shutdown of major MHC class I expression by trophoblast cells appears to be critical for normal placental development and fetal survival. This immunological camouflage is vital to avoid recognition by the multifactorial array of cellular and hormonal mechanisms that mediate rejection. In a mature placenta, maternal immunological recognition of fetal MHC class I proteins triggers immune and inflammatory responses that contribute to rejection of allantochorion at parturition. It is interesting to note that in these situations there is a clear adaptation of the immune system for a function distinct from protection against pathogens. In summary, MHC compatibility has significant molecular consequences and can result in an increased incidence of RFM.

The inflammatory response is followed by the maternal immune response. Two characteristic actions are evident in the inflammatory process, namely production of neutrophil-activating factors and the chemotactic activity of neutrophils. These are important for successful immune-assisted detachment of the allantochorion. It has been suggested that the function of peripheral leukocytes may be reduced, which may be a cause for, or an effect of, retention leading to other complications such as mastitis in RFM cases.

Vascular Changes

During contractions of the uterus, uterine pressure is constantly changing and this leads to alternating anemic and hyperemic conditions and temporary changes on the surface of fetal chorionic villi. As a result, the attachment of chorionic epithelium in maternal crypts becomes impaired. Further, subsequent rupture of the umbilical cord shuts down blood supply to the fetal villi and helps in the separation process by reducing the size of villi. Anemic changes that occur to the fetal villi after calving because of rupture

of the umbilical cord are essential and these mechanical processes of detachment should not be underestimated. Identical changes occur in caruncles due to uterine contractions. Uterine contractions continue to reduce the amount of blood directed to the uterus. Consequently, caruncles become smaller in size due to reduced blood supply, resulting in the dilation of maternal crypts.

Contractile Changes

Abdominal contractions noticed during expulsion of the fetus result in changes to the shape of the placentome due to abdominal pressure, an essential step in the process of separation. Concurrent and intermittent uterine wall contractions change the shape of the caruncles further from oval to round due to fetal pressure on them, facilitating detachment of cotyledons from the caruncle. The characteristic sharp contractions of the circular myometrial muscle layers that closely follow each other and which are superimposed on contractions of longitudinal myometrial muscle layers noticed in the parturition process help not only in expelling the fetus but also in allantochorion separation. These rhythmic waves of contraction (every three to four minutes) cause cotyledons to open up in a fan-like manner as they pass over them and enable separation of the allantochorion. Hence defective myometrial contraction can lead to retention. Progesterone diffusing from the placenta to the myometrium and estradiol synthesized locally at this time may have a role in the initiation and suppression of these contractions. Presence of uterine contractions even after delivery helps in the process of separation. This is also necessary to prevent hemorrhage and to aid in the expulsion of fetal membranes. These peristaltic and contraction waves, besides reducing the size of the uterus and aiding in forcing fetal membranes into the birth canal, can also markedly reduce the amount of blood circulating in the endometrium. Further, the influence of oxytocin and ongoing uterine involution in these processes cannot be overlooked, as noted where injection of oxytocin and suckling had beneficial effects in shedding of fetal membranes.

Pathogenesis

Considering the essential changes that facilitate the process of separation and expulsion of fetal membranes, it is obvious that retarded or unsuccessful separation and expulsion can be due to interference with these changes. Paying attention to interfering factors and eliminating them, if possible, will reduce the incidence. Some of these factors are discussed in the section "Prevention." In general, the interfering factors can be classified as direct or indirect.

Direct Factors

Direct factors include immature placentomes, non-inflammatory edema of the chorionic villi that results from uterine torsion and cesarean sections, dystocia, necrosis between the crypts and villi following a possible antepartum allergic reaction, premature involution of placentomes, hyperemia of placentomes, inflammation, mechanical prevention of expulsion, and inflammation of fetal membrane.

Although inflammation is attributed as a direct interference, the role of prepartum uterine infection is questioned and it is more likely that uterine infection follows after retention due to either poor uterine involution or the fact that the membrane is retained. However, the incidence of retention in infectious abortion cases suggests that infection may be a factor. The most likely reason for retention in such cases may be immaturity of the placentome rather than infection. It should be remembered that poor calving hygiene can lead to parturient infection and retention, although the mechanism is not clearly understood.

In most induced parturition cases, retention of allantochorion can be attributed to possible immature placentomes. However, retention does not occur in all induced cases and furthermore it is influenced by the type of glucocorticoid administered. Besides immaturity, administration of these agents may result in dysfunctional apoptotic processes or interfere with the immune-assisted separation process discussed earlier or specific actions such as the expression of cyclooxygenase by PGF_{2a}. Furthermore, these treatments can inhibit the normal loss of TGC that occurs closer to parturition and modulate the action of other hormones.

Indirect Factors

Indirect factors include intensive stress, duration of pregnancy, extensive distension, season, sex of the fetus, stillbirth, deficiency of trace minerals and vitamins, and situations where the uterus becomes atonic and unable to contract in cases such as dropsy, twinning, fetal gigantism, subclinical hypocalcemia, and other pathological conditions. Other indirect factors that may have a role can be broadly categorized into hormonal, hereditary, nutritional, and circulatory causes; incidentally, these may also contribute to unexplained cases of atonic uterus. The required changes for separation and expulsion of the allantochorion and the interfering factors are schematically summarized in Figure 53.2.

It is not known whether apoptotic, degenerative, and necrotic changes are noticed in placentomal and interplacentomal regions because of allantochorion retention. As mentioned earlier, apoptosis that started prior to separation will continue during retention. It should be emphasized that

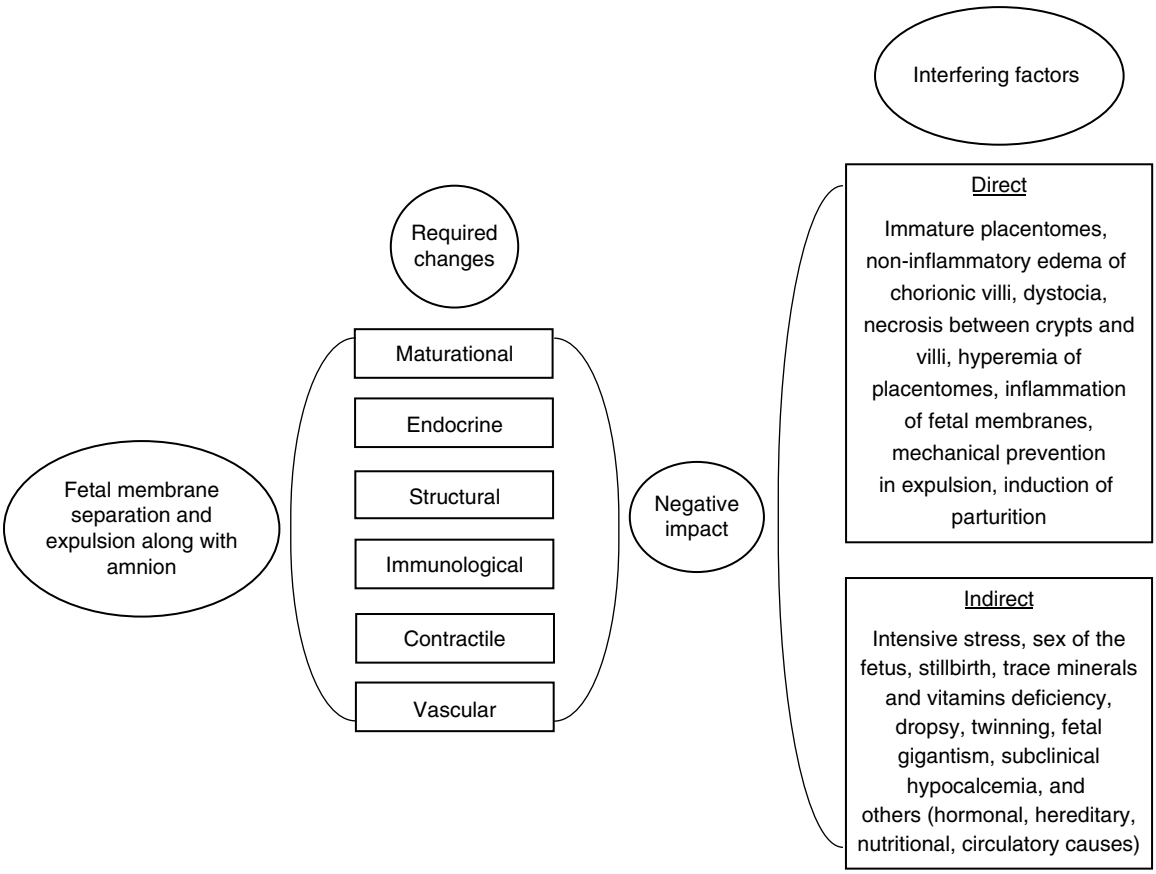


Figure 53.2 A schematic depicting changes required for normal fetal membrane separation and expulsion. Refer to text for details of these changes. Interfering factors that have a negative impact on these processes are listed.

degenerative changes might have been responsible for retention; however, in almost all cases these changes will occur after retention. Similarly, in certain cases small portions of necrotic epithelium present between the chorionic villi and the cryptal wall might contribute to retention, but again the process occurs after retention, depending on the period of retention. In general, the lesion may vary from slight evidence of inflammation to severe necrotic or leathery or hemorrhagic inflammation of allantochorion. Depending on the etiology and its severity, lesions may extend to areas of membrane that occupied the non-gravid uterine horn.

Clinical Signs and Complications

Normally, if fetal membranes are not passed within 36 hours, and in the absence of manual intervention, they tend to persist for 6–10 days as a putrefying tissue and drop away as a putrescent mass in uncomplicated retention. Progress of putrefactive liquefaction of the villi allows separation, and uterine involution aids in completing separation. Some cotyledons toward the cervical end would have separated by the time the calf is born. Involution of the uterus to restore its spiral configuration aids in separation. Mild contractions of the uterus and cervix are accompanied by, and correlated with, transient dilations of the caudal cervix that facilitate evacuation of normal uterine exudate. In protracted cases there is a large amount of uterine exudate with its characteristic foul-smelling odor. Vaginal/uterine examination of a cow in such cases can evoke forceful straining that results in expulsion of fetid uterine exudate along with feces.

In general, depending on the duration of retention, a case can have fresh fetal membranes hanging from the vulvar lips (Figure 53.3) without any obvious smell, or it might be one that has undergone degenerative changes, as reflected by the appearance of fetal membranes and the presence of malodorous stench. There may not be elevation of body temperature with or without marked illness in all cases even if retention has continued beyond 72 hours. Immediate complication may be reduction in milk yield, which may not always be noticeable and the impact may be negligible on the herd basis. Some animals exhibit outward signs of ongoing uterine changes in response to spontaneous uterine infections or uterine infections that followed due to manual removal. Along



Figure 53.3 A fresh case of retained fetal membrane with minimal changes. *Source:* Courtesy of Robert Lofstedt.

with a rise in body temperature, other signs may be obvious such as anorexia, depression, increase in pulse rate, and loss of body weight. These are symptoms of either endometritis or puerperal metritis and the signs may depend on the intensity of the disease process and the number of uterine layers involved in the inflammatory/infectious process. Further, the foregoing clinical signs may be severe if other conditions such as peritonitis, mastitis, necrotic vaginitis, parturient paresis, and acetonemia occur simultaneously with uterine changes.

Apart from immediate complications, long-term complications include delayed uterine involution, minor to major effect on fertility, inherent loss of milk production, or loss due to discarded milk because of antibiotic treatment. The immediate effect on fertility is mainly due to impaired uterine and ovarian activities. There will be an increase in days from calving to first estrus and the occurrence of subsequent estruses. In essence, the resumption of normal cyclic ovarian activities is prevented, possibly due to delay in uterine involution and elimination of bacterial infection. Estrous cycles may be irregular, leading to increases in the number of breedings and finally leading to increases in days open. It is difficult to determine the overall losses; however, considering the ramifications of the condition, it can have a very serious impact depending on its prevalence and incidence.

Treatment

Considering the variety of causes and mechanisms, it is not surprising that treatments have met with limited success. However, more than treatment, a case of RFM, like other clinical conditions of the immediate postpartum period, enables the veterinarian to become an educator more than a curer of all animal ailments. Basic goals of any therapeutic regimen should be return of the cow to reproductive usefulness as soon as possible and the prevention of primary and secondary complications that can lead to economic losses from lowered milk production and delayed conception. The primary complication is a normal uterus becoming a diseased or pathologic uterus and the goal of treatment is to prevent its occurrence.

The aim of treatment is to ensure that RFM are shed within a reasonable time with minimal intervention. It should never be the goal to manually remove by force, except in cases where the allantochorion has completely detached but the membranes are not expelled for any unexplainable reason. In other words, the only time that manual removal should be practiced is when the attending veterinarian fortuitously finds the entire fetal membranes free and expulsion only hindered by a partially closed cervix. In this case the fetal membranes are gently teased through the cervix, with care being taken not to traumatize the cervix or vagina during the procedure. Removal should be completed within a few minutes and carried out gently, paying attention to hygienic measures. Aggressive intrauterine manipulations probably increase endotoxin production and its absorption into the circulation, resulting in elevated body temperature. Intrauterine or systemic antibiotic treatment is not necessary. However, lack of secretion of thick tenacious mucus by the cervix in RFM cases allows infection to gain entrance to the uterus and the process of removal can further favor endometrial infection. Hence some cases may need systemic antibiotic treatment depending on the severity of the problem and condition of the animal.

In general, manual separation of the caruncular and cotyledonary attachment is not a preferred method. Although it appears to have many harmful effects on the animal and on future fertility, it is believed to be practiced for aesthetic reasons. This practice seems to give a "conservative trust to an old routine" and is accepted in many parts of the world and taught in the veterinary curriculum. If it is practiced, the cow's tail should be secured by tying it to the opposite front leg. The vulvar area is then prepared with a suitable antiseptic scrub, and tetracycline boluses or capsules are introduced into the cervix and uterine horns with a clean plastic sleeve after the allantochorion is separated carefully by releasing the attachments between the caruncle and cotyledon. Systemic administration of ceftiofur hydrochloride is benefi-

cial for prevention of metritis. Although some veterinarians prefer a predominantly intrauterine approach for antibiotic therapy to combat uterine infection, a systemic approach has certain advantages. For example, absorption of antibiotics from the uterus is not impaired as in local treatment, antibiotic concentrations attained in the uterine tissue are similar to blood concentrations, and uterine infection is seldom localized since it involves more layers than uterine mucosa.

Dilating the cervix after a couple of days in an attempt to expel fetal membranes is not recommended. This causes more trauma to the cervix and uterus, predisposing to further complications, notwithstanding the pain inflicted to animals. Complications include immediate septicemia, drop in milk and appetite, elevation of temperature, loss of weight, and possible death, or else a prolonged period of convalescence during which time shreds of membrane and exudate are expelled, pyometra or chronic metritis develops, or protracted or even permanent infertility may result from uterine abscess, perimetritis, salpingitis, or severe endometritis. It is a good idea not to examine the cow until 72 hours after parturition unless the cow develops clinical signs of anorexia, an elevated temperature, and other symptoms of septicemia. It should be noted that in some cases it is impossible to insert a hand into the uterus after 48 hours and cervical closure occurs in normal cases earlier than this timeframe. Delaying for 72 hours allows the endometrium to construct a protective barrier by walling the fetal membranes off and thereby lowering the chance of septicemia or toxemia. Autolysis that will eventually occur enables separation and expulsion. In such cases, treat as a large abscess in which the necrotic core has not been separated from the surrounding tissues. At no time should fetal membranes be tied to an object or cut closer to the vulva. Surgical procedures governing the handling of an abscess should also govern the handing of an infected uterus. Conservative treatment is indicated and essential. Removing fetal membranes in an incomplete, rough, and insanitary manner is not advisable. In certain cases, there may be collection of uterine exudate; gentle vaginal/uterine examination allows the fluid to drain out by the position a standing animal would take in response to such an examination.

Uncomplicated cases require no treatment and thereby the impact on future fertility is minimized. A "non-drug approach" to the problem should not be forgotten. However, the malodorous stench generated by retention cannot be ignored and it requires courage to prescribe no treatment, so it would be prudent to adopt a rigid attitude of non-interference. A conservative approach (intrauterine antibiotics, maybe including systemic drugs, and leaving the fetal membranes) is better than manual removal at the behest of the client, since clients would prefer that the unsightly fetid mass hanging from the genital tract is removed earlier.

If fetal membranes are retained for 24–48 hours and the cow is running a fever (rectal temperature >39.5 °C) or anorectic, the animal should receive immediate attention and treatment. In addition to monitoring rectal temperature, close observation of the animal is critical. Nothing replaces close observation that recognizes an animal's changing appearance and behavior. The minimum treatment would be parenteral administration of antibiotics. Systemic treatment alone is effective and intrauterine antibiotic treatment is not recommended in all cases since more harm can be done by the manipulation. For systemic administration, ceftiofur is preferred over oxytetracycline: the latter is approved for beef and non-lactating dairy cattle and has a 22-day preslaughter withdrawal period, while ceftiofur is appealing because of injection-site tolerance, no milk discard, and 3-day preslaughter withdrawal period. Although environmental fragility and residue chemistry of cephalosporin compounds have not been explored completely, these may be a concern with regard to animal and human food safety. If oxytetracycline is used, four days of milk discard is required. Penicillin can also be used provided a similar milk discard time is followed. It should be remembered that there may not be better reproductive performance due to antibiotic treatment, but in general proper antibiotic treatment may reduce the chance of the treated animal being culled from the herd.

In the USA, it is required that all these treatments should be carried out with the knowledge of a practitioner who is aware of the Animal Medicine Drug Use Clarification Act. It is incumbent on the practitioner to ensure that the right dosage, route of administration, and duration of treatment are strictly followed. Clients also need to be clearly instructed about the withdrawal time for milk and preslaughter withdrawal period. Cows that appear depressed and which are dehydrated need further examination and uterine exudate should be drained by flushing. No single course of treatment is safe and effective in every circumstance. Considering the perceived and real effects of the use of antibiotics in animals on human health, intrauterine agents such as chlorhexidine, hydrogen peroxide, and recently ozone have been tried.

Use of ecbolic drugs has been suggested as a way to improve uterine and myometrial contractions to aid in separation and expulsion. Foremost is $PGF_{2\alpha}$, but its use is questioned since the endometrium produces this compound adequately during the immediate peripartum period. The inconclusive nature of the findings supports this notion. Some studies have advocated its use at later times in the postpartum period to improve fertility by regulating the uterine involution process and facilitating early resumption of ovarian activity. However, findings are not uniform, real effectiveness remains undetermined, and most results seem

to deny any positive effect. However, there may be instances where it may have some benefit, particularly in the presence of a corpus luteum that hinders the restoration of uterine health in the face of uterine infection.

Administration of compounds prophylactically or as a treatment option based on their ability either to facilitate separation of the allantochorion or to contract myometrium have been developed over the years and continue to be used. Many other agents continue to be used to reduce the deleterious effects on fertility. The prophylactic use of agents such as antibiotics and estrogens to prevent endometritis and improve fertility is a reasonable goal. Estradiol compounds are used as an adjunct treatment option to aid in restoration of endometrium and prevent metritis. This method has empirical appeal; however, paucity of beneficial information, possible side effects, lack of conclusive scientific evidence, its effect on humans as a food residue, and marginal return considering the time and cost involved limit its universal application. Lack of availability in some parts of the world due to regulatory measures also precludes its use.

Other compounds such as gonadotropin-releasing hormone (GnRH) have been used to improve fertility in the postpartum period with variable results. However, GnRH has benefit when the ovary and uterus are structurally and functionally ready for the initiation of follicular activity and the animal is fit for breeding. In certain herds that practice earlier breeding, GnRH may be beneficial. A small dose and repeated use of oxytocin has more scientific validity for treatment because of its ability to cause tubocervical contraction of the uterus and thereby expel fetal membranes and uterine exudate. Furthermore, for the same reason, its use in cesarean section is warranted to facilitate separation of the allantochorion. The amount used is important since it can enhance milk ejection prematurely. In general, lack of unified opinion has been magnified by endless clinical reports and testimonials concerning the ever-growing list of hormones and antimicrobial solutions. Use of ozone [27–29] may have its benefit, but much research is needed.

Administration of collagenase into the umbilical arteries has been studied extensively and is found to aid in separation of the allantochorion. Although the technique is promising, time, the cumbersome nature of the procedure, and cost limit its use. Further, the effect on future fertility remains undetermined.

It is recommended that all cows affected with RFM be examined 20–30 days after removal of fetal membranes to determine the progress of uterine involution. A good transrectal examination of the reproductive tract not only helps to identify problems, if any, but also stimulates the uterus and enables expulsion of uterine contents. Transrectal uterine massage around this time, starting from the uterine horns to the cervix, for three to five minutes may stimulate uterine mucosa and restore the dynamic function of the myometrium. This procedure is contraindicated in cases of fibrinous, hemorrhagic, acute purulent endometritis and closed cervix cases. Complications of unresolved cases are many and are aggravated by the animal's body condition, age, and management practices. It should be noted that permanent sterility may result due to pyometra and metritis if proper treatment is not instituted. Aged animals may need longer time to recover, so breeding should be delayed. All cows with persistent mucopurulent discharge from the vulva following RFM should be reexamined and treated.

In summary, manual removal of retained membranes is not recommended and each case does not require treatment during or after expulsion of fetal membranes. Systemic antibiotic treatment can be reserved for animals that have fever, while some animals may in addition need fluid therapy. Oxytocin can have beneficial effects if correct dose and regimen are used.

Prevention

Proper treatment of the affected animal will reduce economic losses due to increased calving interval, increased culling rate, discarded milk due to antibiotic treatment, and costs of veterinary treatment and drugs. However, in the modern cattle industry, prevention is more important than treatment in order to augment profitability. It is hard to define the percentage incidence that can be allowed or a percentage that calls for drastic preventive measures. Paying attention to the direct and indirect factors discussed earlier and eliminating these can reduce the incidence, and possibly prevent the occurrence, of RFM. In the interests of animal health and welfare, it is prudent to examine the calving cow and nutrition management, particularly dry-cow nutrition, if the incidence exceeds 10%.

It appears that there is no genetic breed predisposition to this condition; however, repeatability is possible if problems are not addressed. No simple reason can be attributed to the occurrence. Some of the factors involved may be lack of ideal body condition at the time of calving, stress during pregnancy and calving, lack of trace minerals, and lack of a sound vaccination program. Function of the placenta should be maintained during pregnancy and it should preserve its plasticity throughout pregnancy for easy separation. Selenium supplementation may have a beneficial effect in a herd with a regular history of the condition.

One factor that can be addressed is to reduce calving problems. In this regard, selection of bulls based on birthweight expected progeny differences and calf birthweight will reduce calving problems. It should be recognized that these parameters vary in breeds. It should also be remembered that the influence of dam on the calf's body weight is greater than that of the bull. Pelvic measurements and judicial

application to herds can reduce calving problems and will reduce the incidence of dystocia and other complications of parturition including RFM. However, pelvic measurements and their application may have lost their perceived impact due to its feeble effect in reducing the incidence of dystocia.

During the course of pregnancy and parturition, the adverse effects on reproduction should be minimized. In this regard, separation of the periparturient cow and use of straw as bedding appear to be beneficial. One of the preventive measures adopted was to treat every case of retention with antibiotics. This may not be beneficial except in cases that have higher body temperatures, as discussed before. Some of the prepartum peripheral hormonal changes have been suggested to mirror the changes taking place in the placenta that most likely lead to RFM. Despite much research in prepartum endocrine/enzyme parameters to predict the occurrence, there is no current method available to apply in practice. Even if some of the hormonal measurements can help to identify these cases, it is a futile exercise to obtain a sample from each animal and, furthermore, with no clear method available to prevent its occurrence after the prediction, it is not economically justifiable.

Summary

While certain aspects of the condition, such as clinical manifestation and remedial measures, have remained the same over the years, the focus has been on prevention of the condition. This should be the direction in which the dairy industry should move, considering herd health, production, and animal and human welfare issues. It is incumbent on veterinary and allied professionals to improve the health of the cattle population by providing adequate housing and maintaining their body condition during the different aspects of reproduction to prevent this clinical problem.

Dedication

This chapter is dedicated to the memory of the late Professor S.J. Roberts, a founding father of theriogenology, who drew the author to theriogenology by his monumental and exhaustive textbook that he created during the non-computerized age and nurtured this author during his academic career.

Acknowledgments

This chapter would not have been possible without the information gleaned from earlier books and review articles on this and related topics. Only the following are listed in the bibliography and the author acknowledges the scholarship of these researchers.

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54

Postpartum Uterine Infection

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Introduction

After calving, cows need to resume ovarian cyclicity and complete uterine involution in a timely fashion to improve their chances of becoming pregnant in a reasonable time frame. The focus of this chapter is to discuss risk factors, pathogenesis, diagnosis, treatment, and management of primary uterine diseases: metritis, endometritis, and pyometra in postpartum cows. *Metritis* refers to the inflammation of all three layers of the uterus, while cows affected with *endometritis* have inflammation that is limited to the uterine epithelium. Pyometra is the accumulation of purulent material in the uterine lumen, which persists due to the presence of a corpus luteum. Inflammatory conditions of other parts of the tubular genital tract (cervicitis and vaginitis) will also be addressed in this chapter concerning their diagnosis and potential impact on fertility.

According to a 2016 study, the per-case cost attributable to metritis alone in dairy cows was estimated at USD $127–366 depending on parity. This variation in cost was due, at least in part, to a higher differential milk loss in multiparous versus primiparous cows [1]. In general, the economic impacts of uterine infections during the postpartum period are related to treatment costs, milk production losses (actual decline in milk yield and discarded milk), and impaired fertility (embryonic losses). Cows with uterine infections have increased calving to conception interval (hence increased intercalving period) and are more likely to be culled from the herd, thereby decreasing their lifetime productivity.

Risk Factors

The prevailing opinion is that multiple factors are responsible for predisposing a cow to develop a uterine infection during the postpartum period. Dairy cattle are more frequently diagnosed with uterine infections than beef cattle, despite the duration (35–45 days) of uterine involution being the same within the species. The difference in susceptibility to uterine infection is often attributed to metabolic stress due to higher milk production and more intensive management practices associated with modern dairying in many parts of the world. Dairy cattle are also observed more frequently and are more likely to be included in reproductive herd health programs than beef cattle; therefore affected animals are more likely to be flagged for examination and treatment. Data from an older study showed that age-matched dairy cattle produced more milk and lost body weight by 15 weeks of lactation in comparison with their beef cattle counterparts that were fed a similar ration [2]. Further, these dairy cows had increased levels of metabolic stress markers (non-esterified fatty acids [NEFA] and beta-hydroxybutyric acid [BHB]) but lower levels of insulin and glucose than the beef cows during the intensive sampling period. Within the dairy cow cohort, milk yield of >35 l/day was associated with a risk of developing uterine disease compared to those producing <35 l/day even though these two groups of cows had similar genetics, uterine bacterial load, and housing conditions [3].

Calving-related events such as dystocia, twins, stillbirths, and abortions are highly correlated with an increase in the proportion of cows diagnosed with uterine diseases (Table 54.1). Frequently, cows with calving problems also have retained fetal membranes due to interrupted endocrinological and histological processes associated with placental maturation, separation, and expulsion. Undoubtedly, this is why retained fetal membranes are often cited as an important risk factor for the development of uterine infection. Peripheral immunosuppression, negative energy balance (body condition score loss), and metabolic disorders (hypocalcemia and ketosis) occurring during the transition period (three weeks prior to and three weeks after calving)

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 54.1 Risk factors associated with the development of uterine diseases in postpartum dairy cows.

Uterine disease	Risk factor (odds ratio) [reference]
Metritis	• Retained fetal membranes (27.7) [4] • Stillbirth (6.3), twin births (6.6) [4] • Dystocia (2.56–4.32) [4, 5] • Hypocalcemia (1.8) [6] • Clinical and subclinical ketosis[a] (1.75–1.91) [7, 8] • Negative energy balance[b] (1.51) [8]
Endometritis	• Retained fetal membranes (3.9–40.3) [9, 10] • Stillbirth (7.5) [10] • Ketosis (2.2–3.8) [10–12] • Hypocalcemia (3.5) [11] • Dystocia (2.2) [5, 9, 10] • Twins (2.2) [13] • Metritis (1.4–2.3) [5, 13]

[a] Subclinical ketosis: increased BHB levels (>1.4 mM).
[b] Negative energy balance: increased prepartum NEFA levels (>0.4 mM) and postpartum NEFA levels (>1.0 mM).

also increase the likelihood of cows developing the uterine disease during the postpartum period (Table 54.1). Other factors such as parity, sex of the calf, season, and milk yield have been associated with a higher incidence of uterine disease in dairy cows [4]; however, these elements are most likely acting through other predisposing factors identified above. For example, primiparous cows are more likely to develop dystocia and thus are more predisposed to having retained fetal membranes and/or metritis.

Pathogenesis

The pathogenesis of uterine inflammatory conditions has been reviewed in detail elsewhere [14, 15]. Cows in the transition period are susceptible to developing infection due to peripheral immunosuppression [16–18]. The degree of immunosuppression during this period is influenced by the occurrence of calving-related stress; the extent of the negative energy balance which occurs post-calving in dairy cattle; changes in the hormonal milieu; and increased oxidative stress [19, 20]. Following calving, all cows experience uterine inflammation associated with the cleanup phase of uterine involution. The ability of the bovine uterus to resolve this inflammation is dependent upon the balance between uterine defense mechanisms and the microbial population.

Uterine involution is a complex process that includes clearance of cellular debris and bacteria, de-epithelialization and subsequent epithelial regeneration of the caruncles, and a tremendous reduction in size from the pregravid state. Following the expulsion of the fetal membranes, less intense uterine contractions continue during the immediate postpartum period under the influence of prostaglandin F2 alpha (PGF) and oxytocin to expel remaining uterine contents through the cervix [21, 22]. Clinically, this material referred to as lochia is observed as odorless, mucoid, and bloody to reddish-brown-colored discharge on the ground where a recently calved cow was laying, or may be seen adhering to the ventral commissure of the vulva and/or around the tail. The amount of lochia expelled is maximal during the first two to three days post-calving owing to the higher frequency of postpartum uterine contractions at this time. Thereafter, both the amount expelled and the appearance change such that astute observers may see a purulent or mucopurulent discharge by two weeks postpartum; however, it should not be visible after the third week postpartum in normal, healthy cows [23].

The initial postpartum uterine inflammatory response is most likely instigated by degenerating placental tissue, followed by bacteria entering through the vagina [15]. Repeated bacterial recontamination of the uterus continues to occur during the first two to three weeks postpartum due to a patent cervix and a natural vacuum created by contraction and relaxation of the uterus [24, 25]. Longitudinal studies using routine bacterial culture and isolation techniques showed that the proportions of cows with mixed populations of bacteria in their uterine cultures declined progressively: 9/10 cows during the first two weeks; 8/10 between the third and fourth week; 5/10 between the fifth to sixth weeks; and 1/10 between the seventh to eighth weeks postpartum [3, 26, 27]. Bacteria commonly isolated from samples taken from the postpartum uterus included *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella* spp. [3, 26, 27]. These species of bacteria have been reported to work in synergy to populate the uterus, evade uterine defense mechanisms, and cause uterine inflammatory disease. All have a negative impact on subsequent fertility. A preponderance of *E. coli* during the first week postpartum is followed by an increased abundance of Gram-positive bacteria, mainly *T. pyogenes*, during the second week [28]. Evidently, bacterial species prefer certain domains within the uterus, with *E. coli* and *T. pyogenes* on the surface of the uterine epithelium, while *F. necrophorum* prefers the uterine submucosa for growth and propagation [29]. A synergistic association between aerobes (*T. pyogenes*) and anaerobes (*F. necrophorum*) along with the acquisition of specific virulence factors

further potentiates their ability to overcome uterine defense mechanisms and populate the uterus [30, 31]. Certain *E. coli* isolates develop genes that allow them to adhere to uterine epithelial cells, while *T. pyogenes* strains possess adhesion properties and also secrete pyolysin that facilitates penetration by damaging the uterine epithelium [32]. Both *E. coli* and *T. pyogenes* possess the ability to reduce the oxidative burst capabilities of neutrophils, thus creating an environment favorable to sustained uterine inflammation [33]. Recent studies using sequencing techniques based on the 16s ribosomal RNA gene have reported that there may be a more diverse microbiome in postpartum cows that are undergoing normal uterine involution compared to those with metritis and endometritis [34]. Furthermore, this apparent divergence of uterine microbiota was evident as early as seven days postpartum when cows that developed endometritis weeks later were compared to those that had an otherwise healthy postpartum period [35].

At the cellular level, the damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) of cellular debris and bacteria, respectively, are recognized by the pathogen recognition receptors (PRRs) present on the uterine epithelial cells (Figure 54.1) [14, 15]. In response, epithelial cells secrete pro-inflammatory cytokines (interleukin [IL]1α, IL8, IL6) and also stimulate resident macrophages (present in the uterine submucosa) to release additional cytokines (tumor necrosis factor alpha [TNFα]). These cytokines attract neutrophils along with some macrophages into the uterine lumen to limit bacterial growth and propagation through phagocytosis. Uterine epithelial cells also participate in inhibiting bacterial colonization of the uterus by secreting antimicrobial peptides and providing a physical barrier to the entry of bacteria into the submucosa. Between 8 and 14 days postpartum, the caruncular epithelium is sloughed off. Although it is an essential part of uterine involution, the denuded surface is susceptible to infection, necessitating the existence of a highly functional cell-mediated immune system to provide protection during this period [36].

Coincident with the waning of uterine inflammation, reepithelization of caruncular regions of the uterus is completed by 30 days postpartum, thus reinstating the epithelial barrier [36]. Some cows would develop a persistent uterine inflammation (primarily endometritis) beyond the fifth week postpartum, which has been associated with poor phagocytic and oxidative burst capabilities of peripheral neutrophils and macrophages [37–39]. Currently, the reasons for such prolonged impairment of leukocyte functionality are unknown but are likely linked to continued metabolic stress in these cows.

Uterine Diseases

Metritis

Inflammation involving all the layers of the uterus, i.e. endometrium, myometrium, and serosa, is known as metritis. Histopathologically, there is edema of the endometrium (mucosa and submucosa) along with leucocytic infiltration and mucosal sloughing. Edema and granular myofibrillar degeneration in the myometrial layer also occurs and fibrin deposition and hemorrhage may be evident on the serosal surface (perimetrium) [40]. By far, metritis is the most clinically evident form of uterine inflammation in cows and is commonly seen within the first two weeks postpartum, with a few cases occurring in the third week after calving. On average, 20% of dairy cows develop metritis; however, the prevalence ranges from 1 to 41% influenced by breed, geographical location, and herd management practices [6, 41, 42]. A severe form of metritis, often referred to as acute puerperal metritis or toxic septic metritis, may occur within the first week after calving. Acutely affected cows have body temperatures >39.5 °C, an enlarged flaccid uterus, and watery red-brown to purulent discharge with a fetid odor (Figure 54.2), and show signs of toxemia (anorexia, sudden and profound drop in milk yield, recumbency, dehydration, and tachycardia) [43, 44]. Animals with subclinical metritis do not show signs of systemic involvement [43, 44]. Following an episode with metritis, cows have a significant drop (15–20%) in their first service conception rate, an increased calving-to-conception interval by almost one estrous cycle length, and a higher risk of being culled from the herd versus their herdmates that have undergone normal uterine involution [45–47].

Commonly isolated bacterial species from cases of metritis include *E. coli* predominantly, followed by *F. necrophorum* and *Prevotella* spp. Strains of *E. coli* expressing FimH adhesion protein appear to have an affinity for attachment and growth on the uterine epithelial surface [30, 31]. Likely, the early growth of these virulent strains of *E. coli* in the uterus (2 days postpartum) facilitates infection with *F. necrophorum* later (8–10 days postpartum) [48]. By the beginning of the second week postpartum, *F. necrophorum* may have already gained access to the uterine submucosa at the deepithelized caruncles and invoked an inflammatory response. In addition to other virulence factors, *F. necrophorum* secretes a leukotoxin that can lyse innate immune cells [49], to potentiate its own growth and that of other invaders such as *T. pyogenes*. This synergistic relationship has been reported as an essential factor in the pathogenesis of several inflammatory conditions involving different tissues and organ systems [50]. Moreover, these synergisms

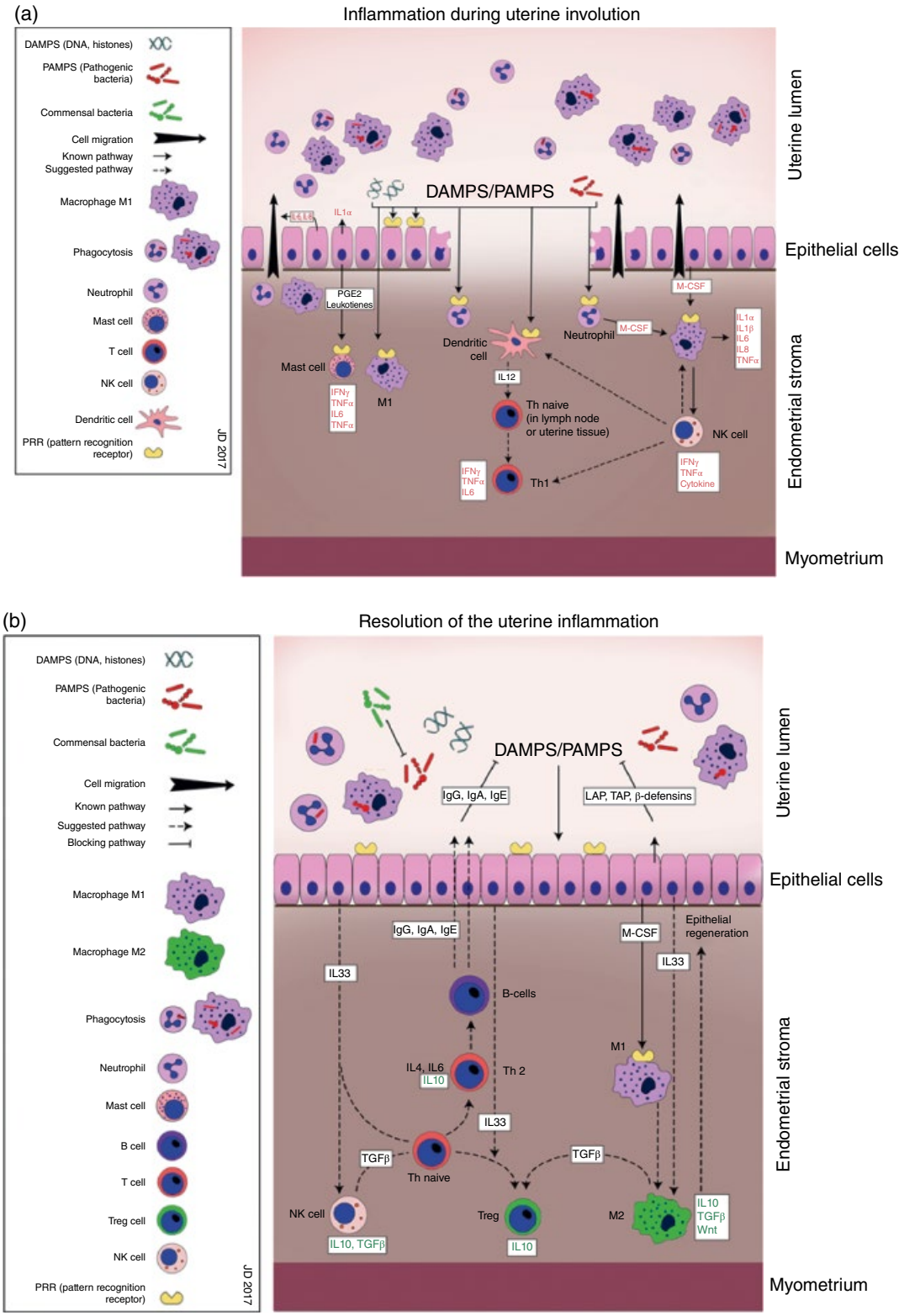


Figure 54.1 Known (solid arrows) and proposed (dotted arrows) mechanisms for the progression (a) and resolution (b) of uterine inflammation in the postpartum cow. Cytokines in red and green text indicate those participating in pro- and anti-inflammatory mechanisms, respectively. (a) Early in the postpartum period, both DAMPS molecules arising from placental separation and tissue degeneration and PAMPS molecules from pathogens and environmental contaminants are recognized by PRRs on uterine epithelial cells. Activation of epithelial PRRs results in pro-inflammatory cytokine and chemokine (IL6 and IL8) release that recruit neutrophils and

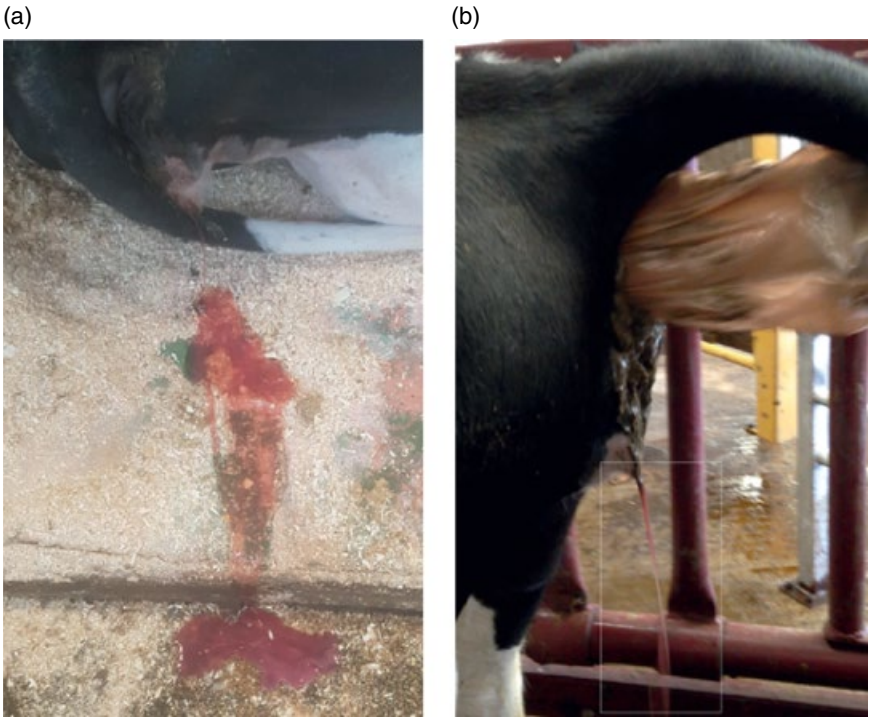


Figure 54.2 (a) and (b) Uterine discharge from cows diagnosed with metritis. *Source:* Courtesy: Gordon Hamm and Dr. Kamal Gabadage, University of Saskatchewan, Saskatoon, Canada.

could be the reason why infection persists in a proportion of cows that are later diagnosed with endometritis.

Diagnosis

Cases of metritis are often presented as cows with a history of calving difficulties and/or retained fetal membranes that have recently become anorectic or dropped their milk production. Often there is an associated elevation in body temperature, now understood to be due to increased levels of endogenous pyrogenic cytokines such as IL1, IL6, and TNFα and/or due to the downstream effects of pathogen binding to their toll-like receptors on the thermoregulatory center in the hypothalamus [51]. A higher than normal body temperature may also be observed during the hot and humid months in many otherwise normal fresh cows [52]. Therefore a thorough physical examination to determine systemic involvement and rectal palpation to assess the uterine status are always warranted. On transrectal palpation, the uteri of cows with metritis are felt as distended and flaccid and observed to lack characteristic longitudinal ridges or "rugae" on the dorsal uterine surface – the sign of a healthy, contracting uterus. Generally, there will be some uterine fluid expelled during palpation. Vaginoscopy to assess and characterize uterine discharge sequestered in the cranial vagina is generally not needed but may be carried out if one is not sure about the nature of the discharge. It is

macrophages to the uterine lumen. Macrophage colony stimulating factor release from epithelial cells activates resident macrophages (M1) to release pro-inflammatory cytokines (IL1α, IL1β, IL6, IL8, TNFα) and assist neutrophils in phagocytosis. Epithelial cells may trigger mast cell degranulation by secreting prostaglandins. The damaged epithelial barrier of the postpartum uterus may allow PAMPS and DAMPS to penetrate into the subepithelial connective tissue and directly activate dendritic cells (DC), neutrophils, and resident macrophages. Activated dendritic cells secrete IL12 that can activate T-cells resident in either uterine tissue or associated lymph nodes to secrete pro-inflammatory cytokines (IFNγ, TNFα, IL6). Resolution of uterine inflammatory processes (b) may involve both epithelial cells as well as a variety of immune cells, including anti-inflammatory macrophages (M2), regulatory T-cells (Treg), natural killer (NK) cells, and possibly commensal bacteria. Epithelial cells may reduce inflammation directly by secreting antimicrobial peptides and fraction 3 of complement and indirectly by secreting IL33. IL33 secreted from epithelial cells and M1 pro-inflammatory macrophages in the later stages of inflammation promotes differentiation of macrophage toward an M2-type phenotype and promotes T-cell differentiation to Treg cells. B-cells respond to bacterial antigens by secreting immunoglobulins which are transported to the endometrial surface to facilitate bacterial opsonization and phagocytosis by neutrophils and macrophages. NK cells initially start with a pro-inflammatory profile and then progress toward an anti-inflammatory profile. Resolution of inflammation may also be supported by IL10 and transformation growth factor (TGF)β secretion from macrophages (M2 phenotype), Treg cells, and NK cells. *Source:* From [15], © 2017, Elsevier.

critical to assess cows with metritis for concomitant mastitis, hypocalcemia, ketosis, or displaced abdomen. These conditions are commonly encountered at the same time and reflect the systemic nature of metritis. The use of ultrasound is not necessary for diagnosing metritis and offers little value other than to visualize fluid and debris in the uterus. A complete blood count (CBC) and blood chemistry typically show leukocytosis with or without a left shift and increased haptoglobin, respectively [53, 54], as well as pain biomarker substance P if testing is available at the lab [54].

Treatment

The treatment of choice for metritis usually includes systemic administration of broad-spectrum antibiotics for curtailing bacterial infection and anti-inflammatory drugs to control fever and inflammation. Spontaneous self-recovery is reported to occur in one out of five cows affected with metritis [55]; however, currently there are no reliable means to predict which animals these will be. Clinicians should be aware that there are strains of *E. coli* [56] and *T. pyogenes* [57] associated with metritis that may be resistant to commonly used antibiotics. A prudent approach to the use of antibiotics might be to avoid antibiotic treatment in the absence of systemic illness and to base the choice of antimicrobial on culture and sensitivity results. Other supportive therapies such as intravenous or oral fluids to correct dehydration, intravenous or oral calcium to address hypocalcemia, and polyethylene glycol to treat ketosis should be administered as warranted.

Both penicillin [58] and ceftiofur [59, 60] are known to concentrate in the uterine tissues following systemic administration at levels that are higher than the minimum inhibitory concentration (MIC) required for bacteria causing uterine diseases. Penicillin administered at 22 000 IU/kg intramuscularly (i.m.) for five days has been observed to resolve metritis in cows [61]. Although the cost of treatment may appear less expensive, the associated milk (4 days) and meat (10 days) withdrawal times must also be considered. A meta-analysis of 23 published clinical trials has indicated that ceftiofur is the most commonly used antimicrobial to treat metritis [62]. Preference for the use of ceftiofur is supported by studies comparing the MICs for the third-generation cephalosporins, penicillin, and tetracycline against the common uterine pathogens [63, 64]. Ceftiofur and other cephalosporins have the lowest MICs against the uterine-specific strains of *E. coli*, *T. pyogenes*, *F. necrophorum*, and *P. melaninogenica*, while tetracycline has the highest MIC [64]. These MICs apparently vary between cows kept in tie-stalls [64] versus those on pastures [63]; however, the clinical significance of such a difference is unknown. Ceftiofur sodium, which is approved for use in dairy cattle at dose rates of 1 and 2.2 mg/kg in Canada/Europe and the USA, respectively, can be administered once daily for three to five days intramuscularly or subcutaneously. At the 1 mg/kg dosage rate i.m. and once daily, peak concentrations of ceftiofur in uterine tissues are reached after the third injection [59]. Therefore it is recommended that at least a five-day treatment protocol is required to treat metritis successfully. A distinct advantage of the 1 mg/kg dose of ceftiofur sodium is that there is no associated milk or meat withdrawal period. A sterile suspension of ceftiofur hydrochloride salt can be also used to treat metritis cases at 1.1 mg/kg (labeled dose) or 2.2 mg/kg (extra-label use) administered daily for five days, with the higher dose reported to have higher cure rates [65]. Alternatively, ceftiofur crystalline free acid at a dose of 6.6 mg/kg may be injected once in the base of the ear if repeated handling and treatment of animals is not an option. A single dose of ceftiofur crystalline free acid is, however, not usually effective for treating metritis; hence a second dose may be administered 72 hours after the first in the base of the opposite ear to improve the cure rate [66]. Appropriate care must be taken while injecting ceftiofur crystalline free acid, as the accidental intra-arterial injection can be fatal to the cow. Since this product is not labeled for the treatment of metritis in cattle, the manufacturer's instructions for appropriate meat and milk withdrawal periods need to be followed.

Intramuscular oxytetracycline is commonly prescribed for treating metritis in cows, yet clinical trials conducted to date have not demonstrated the efficacy of this drug. Furthermore, pharmacokinetic studies have not provided strong support for the use of systemic oxytetracycline. The highest concentrations of oxytetracycline achieved in the uterine tissues are only about a third of the desired MIC for the common uterine pathogens [67]. Another antibiotic that could be utilized to treat metritis is ampicillin, as it is effective against *E. coli* and Gram-positive bacteria [63]. At least one study has shown improvement in the metritis cure rates following treatment with ampicillin trihydrate (11 mg/kg i.m. for five days) [68].

Intra-uterine infusions of antibiotics and other compounds have been used alone or in combination with systemic therapy as treatment for metritis with variable results. A clinical trial comparing repeated intrauterine infusions of 1% povidone-iodine versus a commercial preparation of plant-origin phenolic compound, carvacrol, showed no difference in clinical cure or cow culling rates but lacked a negative control group [69]. Intrauterine infusion of procaine penicillin [68], oxytetracycline [61, 68], and chlortetracycline [70] were observed to have some beneficial effects; however, most of these studies also lacked appropriate negative control groups. Other concerns with intra-uterine treatment are that this route is not approved for most antibiotics; there is lack of

information regarding the required dosage per kilogram of body weight; there is variable tissue penetration from the uterine lumen [67, 71]; and some antibiotics are inactivated by cellular debris and bacteria. Moreover, the duration for which antibiotic residues are detectable in the milk and meat following intra-uterine infusion has not been reported except for procaine penicillin (up to three days in milk) [72] and oxytetracycline (up to eight days in milk) [73–75]. Blanket treatment of cows having retained fetal membranes with intra-uterine antibiotic preparations to prevent or treat metritis should be discouraged. Since most commercially available intrauterine preparations and historically popular veterinary clinic treatments contain oxytetracycline, this approach could result in the propagation of bacterial strains with antibiotic resistance. Similarly, the use of first-generation cephalosporin for intrauterine therapies should be discouraged, as they are not effective for the treatment of metritis.

Uterine lavage to flush the uterine contents using warm sterile saline, tap water, or other liquids is generally not recommended. The risks likely outweigh the benefits, since lavage is believed to increase the absorption of bacterial toxins due to hyperemia within the uterine wall and the escape of toxins into the peritoneal cavity via the oviduct. Uterine rupture can easily occur as a result of overfilling or rupture of the thin uterine wall by the tube, especially when its free end is beyond the clinician's reach.

Hormonal treatments such as oxytocin, estrogen, and PGF have been utilized as the primary or adjunct therapy to treat metritis based on their ability to facilitate the evacuation of uterine contents. Oxytocin receptor presence in the uterus has been reported up to seven days postpartum in cows; however, the functional status of these receptors is unknown [76]. Nevertheless, this period overlaps with the reactivation of ovarian follicular activity and development of the first dominant follicle that could act as a source of estrogen for the upregulation of oxytocin receptors in the uterus. Estrogen exerts its ecbolic effects directly or indirectly through oxytocin receptors [77], is known to increase the blood supply to the uterus due to vasoconstriction, and can enhance chemotaxis [78] and phagocytic activity of leukocytes [79, 80]. Accordingly, estradiol cypionate (ECP) was historically advocated for the treatment and prevention (when used to treat cows with retained fetal membranes without systemic illness) of metritis in cows, but it is not currently approved by the Food and Drug Administration for use in cattle in the USA. Use of ECP at a dose of 4 mg i.m. administered 24–36 hours post-calving to treat cows predisposed to metritis did not result in a reduction in the proportion of cows diagnosed with metritis at a later date [81]. To date, published studies have not shown any substantial benefits associated with treating cows with either oxytocin [82] or PGF [83] as a preventive or curative measure for the occurrence of metritis and there has been no difference in subsequent fertility parameters associated with their use. Although once believed to be beneficial, it is not surprising that various protocols employing PGF for the treatment of metritis have not survived scientific scrutiny, since we now know that cows with metritis already have elevated levels of PGF metabolites (PGFM) compared to cows undergoing an otherwise healthy postpartum period [84, 85].

Endometritis

Cows affected with endometritis have inflammation that is restricted to the mucosal and submucosal layers of the uterus. Unlike metritis, endometritis does not cause systemic illness, but affected animals might have associated cervicitis and vaginitis. New definitions of the disease led to the terms clinical and subclinical endometritis. It is important to recognize that endometritis should not be diagnosed in cows ≤21 days postpartum to avoid falsely classifying cows undergoing normal uterine involution. Classically, *clinical endometritis* has been defined by the presence and grade of the purulent vaginal discharge (PVD) (≥50% pus) in cows that are >21 days postpartum [86]. Discharge should be visualized either in the cranial vagina or at the ventral commissure of the vulva; however, it is noteworthy that PVD may not be observed in all cases of endometritis [87]. Some animals may have a closed cervix (luteal phase) at the time of examination [88], while others might be more effective at clearing uterine discharge. Subclinical endometritis is characterized by the presence of increased proportions of neutrophils in the uterine cytosmears using threshold levels that have been defined relative to the number of days in milk (see below).

Postpartum cows undergoing normal uterine involution processes have a peak infiltration of leukocytes (primarily neutrophils) during the first week postpartum, with neutrophil counts declining to zero by the fifth to seventh week postpartum. As shown in Table 54.1, certain factors can predispose cows to develop persistent endometritis and these may be aided by an inadequate mucosal immune response and synergism between uterine pathogens. In that regard, cows with a weaker uterine inflammatory response (lower neutrophil counts) during the first week postpartum are at a higher risk of being diagnosed with endometritis between five to seven weeks postpartum [89]. One research group reported that cows with subclinical endometritis had reduced proportions of phagocytosing neutrophils and monocytes both in the peripheral blood and in the uterus 18 days after the time of diagnosis when compared to non-endometritic cows [38]. However, the exact reason for this prolonged immunosuppression occurring in cows with

endometritis is unknown. It is possible that some of these cows had a negative energy balance and/or had a previous episode of metabolic disease(s) that could have adversely impacted the phagocytic and oxidative burst capabilities of innate immune cells. Reduced dry matter intake and increased NEFA concentrations in peripheral blood during the postpartum period have been associated with a lowering of immune cell functionality and an increased occurrence of subclinical endometritis in cows [90, 91]. It is noteworthy that the postpartum period is demanding on the cow's energy resources for milk production, uterine involution, and resumption of ovarian cyclicity.

The most common bacteria cultured from cows with endometritis is *T. pyogenes*. Infection of the bovine uterus with *E. coli* during the first week postpartum appears to predispose growth and propagation of *T. pyogenes* at three weeks postpartum. The induction of clinical endometritis in virgin Holstein heifers following intrauterine infusion of *E. coli* and *T. pyogenes* indicates further proof of this association between bacteria [92]. Additionally, the presence of *T. pyogenes* in the uterus at 21 days postpartum is highly correlated with the growth of *F. necrophorum* as well as *P. melaninogenica*, diagnosis of persistent endometritis at the fifth week postpartum, and decreased conception rates [89, 93, 94]. Interactions and interdependence between aerobic and anaerobic bacteria seem to be critical for the development of persistent endometritis. Such a synergistic association and domain-specific propagation of different bacteria have been highlighted by recent metagenomics studies as well [35, 95].

Diagnosis

Clinical endometritis can be diagnosed by the nature of mucopurulent discharge (≥50% pus) present at the vulva or in the cranial vagina in cows >21 days postpartum [86]. Contents present on the floor of the cranial vagina can be visualized either by vaginoscopy (Figure 54.3) or by scooping using either the Metricheck™ device (Simcro Ltd, Hamilton, New Zealand) or a sleeved hand and arm. The Metricheck device has been shown to be more effective as 10–17% more diseased cows were discovered that would have otherwise gone undetected [96, 97]. The belief is that the clinician can more consistently reach deeper into the vagina and is better able to scoop exudate as the device is withdrawn. Visualization of the vaginal contents with a speculum may be limited, especially in multiparous cows whose genital tracts may be suspended more cranioventral, creating a pool just caudal to the cervix where discharge tends to accumulate. Delaying the examination of vaginal contents until 28–30 days postpartum will decrease the number of cows undergoing normal uterine involution from being falsely classified as diseased (false positive) [98]. Dubuc et al. first proposed a broader term PVD to identify the postpartum cows that may or may not have an accompanying endometrial inflammation [99]. Cows identified with PVD include those affected with endometritis or cervicitis (enlarged and inflamed cervix) or vaginitis or a combination of these inflammatory conditions. Nevertheless, the observation of PVD in cows ≥28 days postpartum has been consistently associated with decreased first-service conception rates and poor reproductive performance [99–101].

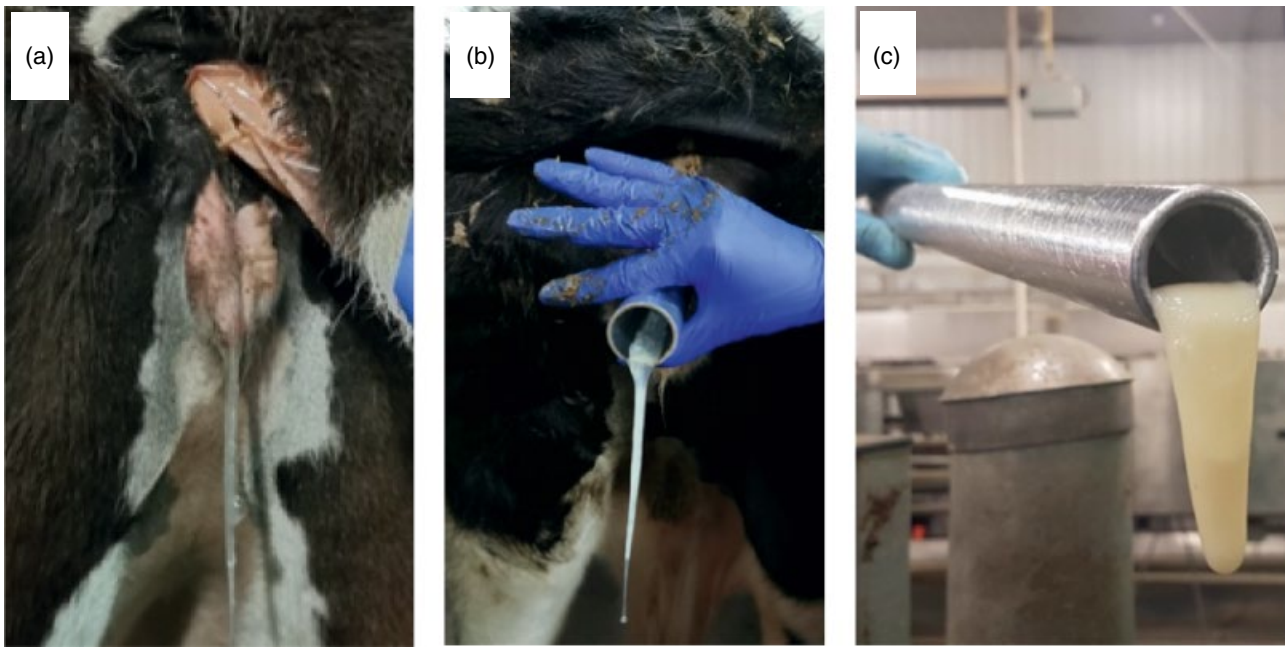


Figure 54.3 Different grades of vaginal discharge visualized on vaginoscopy and on the floor. (a) Slight cloudiness and few pus flakes (grade 1); (b) mucopurulent with 50% pus (grade 2); (c) purulent (grade 3).

Transrectal palpation of the uterus may yield some information regarding the asymmetry of uterine horns, the thickness of uterine walls, and/or the presence of fluid in the uterine horns; however, such information tends to be subjective. In our clinic, a transrectal examination is usually employed to detect cows that should have a more thorough ultrasonographic evaluation of their genital tract. Generally speaking, however, the transrectal examination is not considered to be a sensitive diagnostic tool [99].

Evaluation of uterine fluid volume and echotexture is beneficial for identifying cows with clinical endometritis (Figure 54.4). Most often, these cows will have echogenic inflammatory fluid accumulated at the tips of the uterine horns or in the most dependent part of the uterine horns. Small amounts of inflammatory fluid can be easily displaced by the pressure of a hand and probe, resulting in false negatives. If the cervix is open, the inflammatory fluid line can sometimes be seen to continue right into the cranial vagina. A recent study has indicated that concomitant use of the Metricheck device and ultrasonographic assessment of uterine contents might help decrease false negatives and false positives, respectively; however, this observation needs to be tested in a bigger dataset [102].

Histologically, endometritis is characterized by epithelial damage, infiltration of leukocytes (neutrophils, macrophages, and lymphocytes), the formation of lymphoid aggregates, and, in some instances, ablation or fibrosis of endometrial glands [87, 103]. The degree of these inflammatory changes indicates the severity or chronicity of the disease [104]. Uterine biopsy provides more information than cytology regarding the extent of the inflammatory response in the deeper tissues; however, a single biopsy sample is not representative of inflammatory changes involving the entire uterus and has a longer turnaround time for results. Studies have been equivocal regarding concerns of a potential negative impact that uterine biopsy may have on subsequent fertility [98, 105, 106].

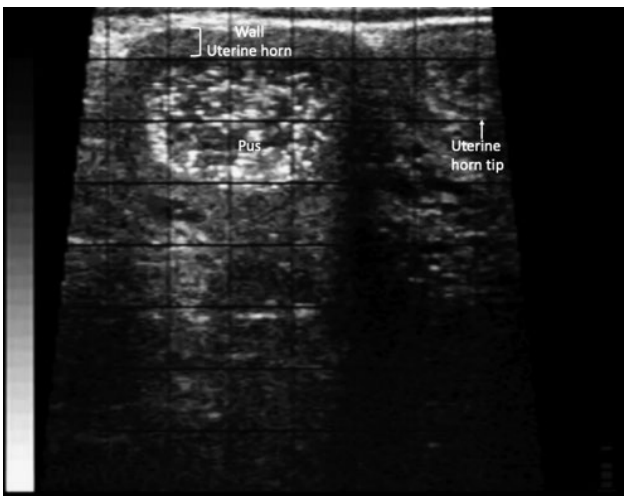


Figure 54.4 Ultrasonographic image of a dairy cow diagnosed with clinical endometritis 28 days postpartum. This cow had purulent discharge adhering to the vulva but there was no corpus luteum on either of the ovaries. Hyperechoic spots in the uterine contents indicate trapped air-bubbles as the cow was scanned after obtaining cytobrush samples to confirm increased neutrophils. Each gridline square equals 1 cm^2.

Since the early 2000s, fundamental research and clinical trials have demonstrated the immense value of uterine cytology as both a diagnostic tool and a means of improving our understanding of the pathophysiology of postpartum endometritis in cows. Uterine cytological samples can be obtained by low-volume lavage [107], cytotape [103], or cytobrush [88, 108]. Harvested samples are then smeared onto microscope slides, dried, and stained using modified Wright Giemsa stain or other commercial stains used for the differential blood cell counts. The proportion of neutrophils is determined by counting their number in relation to the number of other leukocytes and endometrial cells. It is important to exclude the endometrial cell casts from counting to avoid skewing the results. Several techniques have been described for counting; however, for the clinician, determining the proportion of neutrophils in a single 100-cell count of a well-prepared smear will be sufficient. The low-volume uterine lavage method has the benefit of getting a sample that is representative of the entire uterus; however, recovered cells are often distorted due to osmotic damage [107]. A uterine infusion pipette attached to a catheter tip-syringe is passed through the cervix into the uterine body. Once in place, 20–40 ml of sterile saline is infused, followed by gentle massage of the uterine horns to distribute the fluid. Next, using gentle manipulation of the infusion pipette, as much fluid as possible is reaspirated back into the syringe. The recovered fluid is centrifuged, the supernatant removed, and the sediment is used to make a smear on a microscope slide. Some cows may pose a challenge with the recovery of fluid with this technique [108].

Ready to use, double guarded cytobrush devices are available for veterinary use; however, in research settings we have learned to prepare our own. Our cytobrush device preparation first involves shortening the plastic handle of a 15-cm-long brush device designed for human use to enable it to be attached to a guarded stylet of a universal artificial insemination gun or similar device. By doing so, both the length and rigidity of the assembly are improved substantially. The assembly is then guarded using an infusion pipette and a plastic sleeve [88]. Once prepared, the cytobrush, stylet, and infusion pipette will survive sterilization in the autoclave. It is essential to withdraw the cytobrush into the outer barrel of the insemination gun to avoid any distortion of the plastic handle of the cytobrush due to autoclaving. The commercially available plastic sleeves, designed to provide protection when placed over insemination guns, can be applied

over the infusion pipette guard to provide an additional layer of protection just prior to use. The prepared assembly is passed into the uterus, similar to performing artificial insemination in cows. The stylet is pushed forward to introduce the cytobrush into the uterus, then is given two full 360° turns to brush off cells from the uterine wall and before being withdrawn back into the guard and removed from the uterus. It is important to note that no additional digital pressure should be applied to the cytobrush while it is being rotated to avoid excessive tissue damage and blood in the sample. Smears are made by gently rolling the cytobrush onto a slide and cytosmears are air-dried before staining.

A range of threshold proportions of neutrophils from 5 to 18% coinciding with the number of days postpartum when the samples are harvested have been used to identify cows with endometritis [88, 89, 107–109]. Despite the differences in techniques, smears obtained using cytobrush and low-volume uterine lavage have been shown to result in similar cell counts [108]. Our research and that of others supports a threshold of ≥18% neutrophils for samples collected between 28 and 35 days postpartum and ≥10% for samples collected after 35 days postpartum [88, 107]. The use of higher thresholds has decreased the risk incidence of false positives. Furthermore, these higher thresholds have been more consistently associated with increased gene expression of pro-inflammatory (IL1β, IL8, IL6, IL17α, TNFα) [88, 110] and regulatory cytokines (IL10 and IL1 receptor antagonist), again supporting the higher neutrophil threshold as a valid indicator of disease [88]. Despite these benefits, cytobrush cytology cannot yet be touted as a cow-side diagnostic test for endometritis due to the time required for cytobrush preparation, sampling, staining, and reading slides. Despite these limitations, it can be a handy tool to survey herds with reduced reproductive performance. The utility of cytobrush cytology in the future may evolve with modification of the assembly to harvest uterine secretions for the detection of inflammatory cytokines [111] or the detection of novel metabolites.

Treatment

Current treatment strategies for postpartum endometritis in dairy cattle employ PGF and intrauterine infusion of antibiotics. Clinical cure rates following these treatments have varied between studies, which have often been confounded by the definition of disease and clinical cure, the number of cows per treatment group, the timing of treatment, and the occurrence of spontaneous resolution. The proportion of cows undergoing spontaneous disease resolution between five and seven weeks postpartum can vary between 50 and 70% [10, 88, 112], which makes a comparison of the clinical efficacy for any treatment challenging.

Cephapirin benzathine, a first-generation cephalosporin, is the only antibiotic that is approved for intrauterine infusion in dairy cows for the treatment of endometritis, but not in all countries. The drug is available as a 19-g dose in a syringe containing 640 mg of cephapirin (Metricure™, Merck Animal Health) and has two-day meat and zero milk withdrawal period if used according to label instructions. In two large randomized clinical trials, postpartum cows, 28–42 days in milk, diagnosed with endometritis (PVD and/or cytological evidence of disease) showed an improvement in first service conception rates of 8–15% compared with their untreated counterparts [113, 114]. Other studies reporting that intrauterine cephapirin treatment did not improve the overall reproductive performance of cows were most likely confounded by the inclusion of PGF for presynchronization or by the timing of the intrauterine antibiotic treatment relative to the stage of the estrous cycle. It is noteworthy that intrauterine infusion of antibiotics in cows during the luteal phase may not be as effective as treatment during the proestrus and estrus periods. Steroid hormones are known to modulate innate immune responses. Since progesterone is immunosuppressive, the luteal phase would naturally be a supportive period favoring the colonization of bacteria in the uterus [92, 115]. In our practice, endometritic cows in the luteal phase are treated with PGF, followed by the infusion of Metricure three days later. These cows are marked for follow-up at the next herd health visit two weeks later. A second dose of cephapirin in 14 days is advised by the manufacturer; however, we have rarely observed a need to repeat the treatment (unpublished data).

Since the 1990s, single or double doses (14 days apart) of PGF have been used to treat uterine infections owing to PGF's ability to induce luteolysis [116] and uterine muscle contraction [117]. Induction of estrus brings about an estrogen-dominant hormonal status, enhancing neutrophil chemotaxis, mucus production, and uterine contractility, thus allowing immunological as well as physical clearance of bacteria from the uterus [115]. Initial meta-analysis on the use of PGF in postpartum cows proved an association with fewer days open; however, the first service pregnancy rates did not differ between treated and untreated cows [118]. A subsequent meta-analysis of published trials showed that PGF treatment of endometritic cows did not improve their reproductive performance [119, 120].

Non-specific immunostimulants such as recombinant granulocyte colony-stimulating factor and probiotic bacteria [121] need to be tested using more extensive randomized clinical trials to assess their benefit in the prevention and treatment of endometritis in postpartum cows. Such treatments, when available, would allow us to further minimize the development of antibiotic resistance in uterine pathogens.

Pyometra

Accumulation of purulent material in the uterus, presence of a persistent corpus luteum on one or both ovaries, a closed cervix, and anestrus are characteristic signs of pyometra in cows. The reported incidence of pyometra in postpartum dairy cows is less than 2% [122] and, in most cases, is believed to be a sequela to ovulation early in the postpartum period – at a time when many cows are still clearing the uterus of pathogens. Despite an increase in the serum levels of proinflammatory cytokines (TNFα, IL-6) and acute phase proteins (serum amyloid A and haptoglobin), cows with pyometra are not systemically ill [123]. The combination of bacterial synergism, altered immune defense mechanisms, and a closed cervix, due to the presence of corpus luteum, leads to the development of pyometra. A progesterone-dominant hormonal phase always favors the development of uterine disease. The life span of the corpus luteum in naturally occurring cases of pyometra is difficult to determine; however, artificial induction of pyometra resulted in luteal phases lasting >30 days [124, 125]. Both PGF and PGFM were shown to be elevated in the uterine fluid and peripheral blood, respectively, yet the corpora lutea persist. Presumably, the extended life span of the corpus luteum in pyometra cases is due to increased concentrations of prostaglandin E2 (PGE2) in the uterus, which being luteotropic is believed to override the luteolytic effects of endogenous PGF [123, 124, 126]. The exact source of the PGE2 is unknown but could be leukocytes, bacteria, or the uterine epithelium. Nevertheless, progesterone creates a favorable environment for bacterial growth and propagation in the uterus.

Commonly isolated bacteria include *T. pyogenes*, alone or in combination with the Gram-negative anaerobes, *F. necrophorum*, and *P. melanogenica* [125]. Typically, *T. pyogenes* is located in the uterine lumen or on the uterine epithelium, whereas *F. necrophorum* and *P. melanogenica* prefer to invade the endometrium, indicating synergism [127]. Cows with pyometra appear to have a decreased proportion of innate immune cells in both peripheral blood and the uterus [128]. This shift could be a function of bacterial synergism and progesterone-induced immunosuppression.

Diagnosis

Cows with pyometra are often diagnosed during routine herd health visits as having a fluid-filled uterus, no vaginal discharge, and a corpus luteum. Most often, only one uterine horn is involved, but the condition may involve both horns, particularly in long-standing cases. Usually, the wall of the uterus is palpably thicker early in the disease process and fluid can be pushed from one horn to another. An active inflammatory process likely causes the thickening of the uterine horns. As the disease progresses, the purulent material continues to fill the entire uterus, stretching out the uterine wall. An ultrasonographic examination will confirm the presence of a fluid with varying echogenicity, a corpus luteum, and the absence of pregnancy (Figure 54.5). Varying echogenicity is best described as fluid (black) with cellular material (gray speckles), which is very characteristic of purulent exudate. The presence of a fluid-filled uterus may be confused with the uterus of a pregnant animal; however, the cardinal signs of pregnancy – amniotic vesicle, fetal membrane slip, placentomes, and a fetus – are absent. Histologically, the endometrium shows signs of chronic inflammation (chronic endometritis) [125, 127] with or without the involvement of the distal oviducts (salpingitis) [127].

Treatment

The vast majority of cases of pyometra in cows will respond to a single luteolytic dose of PGF (dinoprost tromethamine, 25 mg, i.m.) or its analog (cloprostenol 500 mcg, i.m.). A PVD may be observed within one to two days of treatment. In our clinical experience, and as reported elsewhere [129], approximately 15–20% of cows treated for pyometra may need a subsequent treatment. Following successful treatment (clearance of purulent material from the uterus), roughly 65% of these cows conceived, requiring an average of 2.2 inseminations to become pregnant [129]. In our practice, cows with pyometra are administered two doses of PGF 14 days apart. With reproductive herd health visits at biweekly intervals, the timing of the second injection is coincident with a follow-up assessment to determine the effectiveness of treatment. Further, two induced estruses following PGF treatments facilitate the clearance of pathogens through an open cervix and by the activation of uterine defense mechanisms

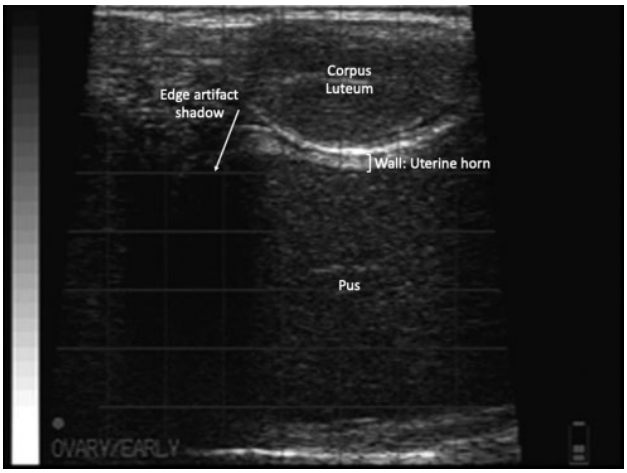


Figure 54.5 Ultrasound image of a dairy cow diagnosed with pyometra at 125 days postpartum. Each gridline square equals 1 cm^2.

during the estrogen-dominant phase. Usually, these cows would conceive following the second to fourth insemination (unpublished data), which suggests that these cows need time to resolve associated endometritis and/or salpingitis before the uterine environment is healthy enough to sustain embryonic development.

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55

Surgery to Restore Fertility

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Introduction

For cows of genetic merit or perceived to be of high value, urogenital procedures are typically performed with the goal of restoring fertility. However, a desire to extend the life of the cow so that a calf can be reared or a lactation completed, or simply to add condition prior to sale often justifies attempts at surgical correction as well. Additionally, while the ability to conceive and maintain a pregnancy and calf continues to be a standard measure for fertility, current assisted reproductive technology provides the opportunity to harvest embryos or oocytes from cows with varying degrees of damage to the caudal urogenital system or uterus. Thus, an attempted surgical procedure that maintains the viability of the ovaries, even if not successfully correcting the injury, is often warranted. The procedures that will be described can be performed on the standing animal with regional analgesia and sedation.

Repair of Injuries Occurring from Dystocia

Parturition is clearly one of the most dangerous events in the life of an animal, whether the dam or the offspring. Common injuries include trauma and tears of the uterus and caudal urogenital system. Tearing of the uterus, whether spontaneous or iatrogenic, can be life-threatening. At the very least, the resultant fibrosis and structural incompetence from perineal body, cervical, and vaginal tears typically results in infertility. Identification of the severity and the provision of a realistic prognosis are critical to the producer, so that an informed decision can be made with respect to treatment versus culling or even immediate salvage.

Repair of Uterine Tears

Most injures to the uterus are iatrogenic and occur during efforts to relieve dystocia. With few exceptions they occur on the dorsal aspect of the uterus (cranial to the cervix) when the obstetrician is attempting to repel a breech presentation or during the forced extraction of a large fetus. In the first case, repair is secondary to removal of the calf. If a cesarean section is performed, the uterine defect may be approached and repaired after the calf is delivered. This is almost always the case when dealing with a tear that occurred during repulsion of a breech position fetus. If the calf can be delivered vaginally following this type of tear or, as in the second case, in which the tear occurs during the delivery, a "blind" suture repair through the vagina can be performed.

I will first examine how these tears occur. In the case of the tear caused by "excessive" force in the obstetrician's attempt to repel the calf, the uterine wall was probably weakened by some level of pressure (ischemic) necrosis. This occurs when the tailhead of the fetus in the breech position is pressed for hours against the dorsal uterine wall. In the second situation, a large calf, a "dry" birth canal, and forced extraction combine to create an overfolding and subsequent shearing of the uterine wall. These tears are usually 5–30 cm in length, although some seem almost circumferential.

Regardless of the tear's etiology, repair may not be necessary if it is small and dorsal. Because of rapid uterine involution, tears smaller than the width of a hand will usually be satisfactory without repair. Treatment for these can be limited to repeated oxytocin injections for the first 24–48 hours and 7–10 days of antibiotics. If the tear is larger, it can be closed with a "blind" suture technique [1].

To perform a one-handed blind closure of a uterine rent, first thread an atraumatic needle (size and shape determined by preference) with 150 cm of #2 or #3 catgut. With

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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the needle at the halfway point of the suture (doubled), make a knot at the end (knot 1) and about 25 cm from the end (knot 2). Next introduce the needle into and through the vagina, guarding the point with the fingers until the tear is reached. Begin closure at one end of the tear. After piercing the uterine wall (about 1 cm lateral to the tear) run the needle between the doubled suture and cinch at knot 2 (the only purpose of knot 1 is to keep the two loose ends together). Then close the tear with a continuous pattern until the last bite is made 1 cm lateral to the other end of the rent. It is important to pull the suture tight with each throw. Then reverse and continue the closure back to the original site and tie to the suture tail. Uterine involution serves to cover small appositional problems. Recommended treatment includes antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), and oxytocin (Figures 55.1–55.4).

Alternatively, the uterus can be prolapsed to facilitate suture repair. To prolapse a uterus, the cervix must still be completely dilated. Therefore this procedure must be performed within 6–12 hours of parturition. Administer 10 ml of 1 in 1000 epinephrine intravenously very slowly. Traditionally, it was advised to dilute the epinephrine in a minimum volume of 250 ml saline. This does not appear to be necessary. Do not perform an epidural or administer oxytocin. The cow must be able to strain and the uterus must be flaccid. Immediately following administration, enter the vagina with an arm, reaching as far cranial as possible to grasp a placentome and pull slowly. This will invert the tip of a uterine horn and the presence of the arm along with the invaginated uterus will stimulate straining. Pull with the cow's straining, grasping other placetomes if necessary. The uterus will usually come out within minutes. At this time, if the rent or tear can be visualized, it can easily be repaired. The uterus is then cleansed and replaced.

It should be noted that this procedure has limited application in that many of the iatrogenic tears encountered as a result of, or during the management of, dystocia are very close to the cervix; after the uterus is prolapsed, the area of the tear will usually still be within the vagina, thus not allowing the accessibility desired. Additionally, some if not most of these tears are near circumferential, and manual prolapse may be synonymous with uterine extraction – because the uterus is held in place with only a small amount of tissue, upon prolapse it simply tears and falls off. Therefore careful evaluation of the tear and its location and severity is warranted prior to prolapse for repair. As with the "blind suture" repair, antibiotics, NSAIDs, and oxytocin should be given postoperatively.



Figure 55.1 With the suture "doubled," the first bite is made at the lateral edge of the wound most easily accessed.

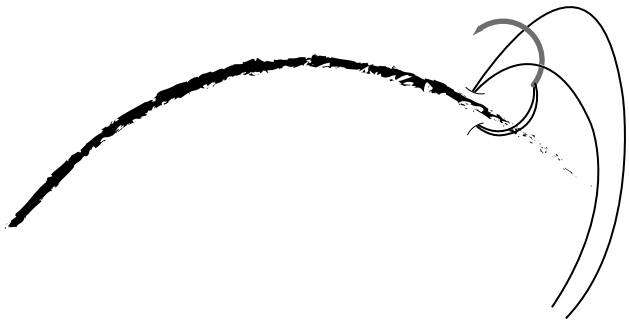


Figure 55.2 After first suture bite is taken, pull the needle through the loop created in the suture (this is not a knot, the suture is cinched).

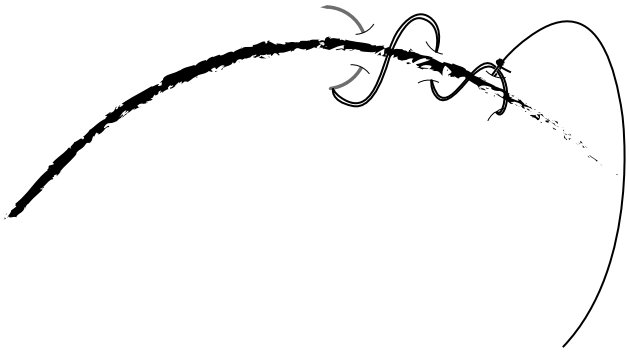


Figure 55.3 Continuous suture bites.



Figure 55.4 After reaching the end of the wound, return. This would look similar to a cruciate pattern and serves to correct the appositional deficiencies of the first row of sutures. When the pattern is complete, the suture is tied to the "tail."

Occasionally a cow will present two or more days following parturition with signs of peritonitis, depression, and anorexia. The cow may or may not have a history of obstetric intervention or even suspected dystocia. Examination may reveal a distal uterine tear which is likely the result of a spontaneous tear or, of course, an injury similar to the iatrogenic injuries previously described. Additionally, the peritonitis could be a complication following cesarean section. In any case, because of the duration of injury and peritonitis, surgical exploration of the abdomen via a flank approach should be performed. Treatment of the peritonitis begins with thorough abdominal lavage, which in turn is facilitated by the ventral placement of a drain.

Utilization of a rumen trocar placed from within the abdominal cavity at the ventralmost aspect will provide very efficient egress for the volume of fluid that is required (Figures 55.5 and 55.6). Also, because of the "siphon" effect that is created, debris (fibrin clots, necrotic material, and placental remnants) will be "pulled" to the drainage site and can be more efficiently retrieved. The rent in the uterus can then be repaired, the uterus lavaged, and finally the flank incision closed. This of course is accompanied by a guarded prognosis, so this level of effort is typically reserved for the cow of genetic merit or the involvement of a highly motivated owner.

Injury to the Vulva, Vagina, and Cervix

Tearing of the vulva, vagina, and cervix occurs almost exclusively during dystocia. Tearing and bruising can often occur during parturition in the absence of obstetric intervention; however, injury to these structures typically occurs during obstetric management of dystocia.



Figure 55.5 A rumen trocar placed from within the abdomen and directed outward at the ventral most location.



Figure 55.6 Fluid flow through the trocar cannula from the abdominal lavage.

Tearing of the vulva during the management of dystocia can be prevented by performing an episiotomy. This is an incision typically made at the dorsolateral (10 o'clock or 2 o'clock) aspect of the vulva. This incision will serve to either avoid or, at the very least, "guide" the tearing that occurs with the extreme stretching of the vulva during dystocia due to an oversized fetus. If an episiotomy is not performed, the tear will most likely occur at the most dorsal aspect of the vulva toward the anus and this in fact is the mechanism for grade 1, 2, and 3 perineal lacerations, a grade 3 being a rectovaginal tear.

Lacerations that involve only the vulva and even episiotomy incisions can be left unrepaired especially if the cow is to be culled. However, this often results in compromised vulvar competence, so these tears should be sutured immediately. A modified vertical mattress pattern can be used. Take a deep bite that includes the skin, fibrous tissue, and mucosal layers, and then the skin only for the superficial bite [2]. Although non-absorbable suture material is often recommended, #1 chromic gut can be used.

Vaginal tearing usually occurs in overfit heifers during the forced extraction of a calf. In these heifers, vaginal fat serves to decrease the cross-sectional area of the birth canal, thus serving as a contributing factor in the dystocia. During parturition, and specifically forced extraction, the vaginal wall will split, allowing the protrusion of this fat. This fat can and should be removed, but suturing the laceration is neither necessary nor recommended [2].

Occasionally the bruising that occurs during parturition will be so severe that the vaginal tissues will become necrotic. Depending on the severity, systemic illness will follow, but at the very least the cow will display tenesmus, a vaginal discharge, discomfort at urination, and vaginal prolapse. Treatment depends on severity and ranges from systemic antibiotics and vaginal lavage to surgical debridement of the affected tissue.

Trauma to the vagina can also result in severe and extensive adhesions. These can occur following dystocia or as a complication of vaginal or cervico-vaginal prolapse. If the adhesions completely occlude the vagina, fluid of a mucoid consistency will accumulate proximally. If this fluid has filled the uterus (mucometra), this is indicative of likely damage to the endometrium and therefore a poor prognosis for future fertility. If the condition is of short duration or if the uterus is not distended, attempts at treatment are warranted. Utilizing epidural anesthesia, insert a gloved hand into the vagina and attempt to break down the adhesions manually. The difficulty with which this is done and the depth of the lesions are prognostic indicators (deeper or more cranial lesions indicate poorer prognosis). Following this, the vagina should be lavaged with several liters of dilute carboxymethylcellulose.

Ensuring that the vagina remains patent after the adhesions are broken down is the key to successful resolution. An inflatable rubber beach ball (25 cm long deflated) with a grommet on one end has been recommended as a pessary [2]. Using #3 Braunamid suture, fix the grommet of the well-lubricated (petrolatum jelly and antibiotic ointment) deflated ball to the cervix. Then slightly inflate the ball. To ensure retention, the vulva is closed with vertical mattress sutures or prolapse pins. Remove the ball in 7–10 days, but continue the application of topical antibiotic ointment until healing. When a ball as described is unavailable, I have used a reusable latex palpation sleeve. The sleeve, closed at one end, is placed in the vagina, filled with loose cotton, and fixed in place.

Injuries to the cervix occur during dystocia and most are iatrogenic. If this is identified immediately, as in the case of an iatrogenic uterine tear or an extension of a uterine tear, it is managed basically as the uterine tear (one-handed "blind" suture). However, many injuries to the cervix are not noticed until artificial insemination (AI) is attempted. These injuries are for the most part not as amenable to correction as those that occur in mares. However, I have examined several cows with cervical injuries or scarring that did not conceive via AI but became pregnant after natural mating. Interestingly, cervical structure following the subsequent calving and involution appeared normal and the cows were again able to conceive following AI.

Rectovaginal Tears

Also termed third-degree perineal laceration, a rectovaginal tear exclusively occurs as a sequela to dystocia. It can be iatrogenic, but most occur naturally due to fetal oversize. In the cow, the constrictor vestibule muscle and cervix are able to provide an adequate anatomic barrier to prevent uterine contamination and infection [3]. Indeed, most cattle with rectovaginal tears or fistulas will continue to conceive and carry pregnancies to term.

Cases in which surgical repair is advisable would be the cow that has been presented as open after attempts at breeding or cows of high genetic merit, specifically the embryo donor cow, for which the owner/manager does not wish to leave the results of future use up to chance. In any case, allow at least four to six weeks following the injury before attempting repair [4].

Several techniques have been described for use in the mare and any of those would be appropriate. The procedure I have used and will describe involves the separate closure of the rectal mucosa, the perineal body, and the roof of the vagina much the same as a technique recently described for the mare [5]. Unlike the mare, the cow has typically soft manure, so dietary alterations prior to surgery are unnecessary. Likewise, it is a matter of choice whether to use a "rectal tampon," a stockinette (diameter 7.5 cm) filled with cotton and placed in the rectum cranially to the surgery site. Regardless, catheterize and empty the bladder, evacuate the feces from the caudal rectum, and administer epidural anesthesia. Alternatively, it is possible to utilize one of the regional blocks described in Chapter 17. Sedation depends on the cow's behavior. The restraint utilized can be either a chute or stanchion. The tail is tied to the side and the surgical site prepared. After cleaning the area, lavage with dilute Betadine solution and dry. I prefer Betadine scrub and solution for this area and do not use chlorhexidine-based products. The use of a chlorhexidine product is discouraged as these products are irritating to the vaginal mucosa and can result in luminal adhesions.

A transverse incision is made along the scar and deepened to a depth of 1–2 cm, carefully dissecting the newly formed "flaps" away from the lateral aspects of the vaginal wall. With careful attention to suture placement in the cranial-almost aspect, begin suturing the roof of the vagina. Various suture patterns and suture materials have been recommended for this procedure in mares, but a simple continuous pattern utilizing #1 chromic gut is adequate. The rectal mucosa should be closed with an interrupted horizontal mattress pattern in such a way as to avoid suture exposure in the rectal lumen. As the closure of the rectal mucosa is advanced caudally, stop every 3–5 cm to place a suture in the perineal body. This effectively closes the "dead" space as you go. The skin is closed with a vertical mattress suture pattern and a non-absorbable (Braunamid) suture.

The advantage of this technique over others, specifically the "6-bite" closure of all layers with one suture, is that it seems to create a thicker, stronger shelf. While this procedure is tedious, the results are usually very rewarding and, as alluded to earlier, it is a procedure reserved for an animal

of high value. Postoperative care consists of antibiotics and NSAIDs for five to seven days, as well as care during transrectal palpation for the next two to three months and perhaps attention at the subsequent calving.

Management of Iatrogenic Injury from Artificial Insemination or Embryo Transfer

Because of the physical structure of the vagina and cervix, iatrogenic injury to the vagina and cervix during the course of AI or embryo transfer (ET) is unlikely or at least rare. However, the uterus and specifically the endometrial lining are damaged easily. The most common injury related to AI is probably uterine puncture following passage of the AI rod completely through the cervix and aggressively continuing anterior passage. This injury in typically related to lack of experience of the technician, but is likely on the increase due to the utilization of "deep-horn" AI when utilizing semen with low sperm numbers, specifically sex-sorted semen or semen that has been evaluated and determined to have low number or low post-thaw motility. Another problem occurs when a straw is broken and the straw or portions of the straw are left in the uterus. Injuries from ET are also typically associated with the novice and include overextension of the Foley balloon, which results in rupture of the endometrium, or stylet-related uterine punctures which would be similar to those created by AI pipettes.

Because it is hard to ascertain how often punctures occur, little is known about the incidence of resultant infertility. Although these injuries likely heal successfully without intervention, a reasonable management tactic when presented with such a case would be a low-volume antibiotic infusion following the injury and then to "short-cycle" the cow [administration of a prostaglandin (PG)]. This would be done 7–10 days later following the AI attempt or immediately if occurring during ET. Cows managed such have conceived on the resultant estrus (note that it is not recommended to breed a cow on the next estrus following an ET flush, so this is applicable only for AI-associated injury).

A fragment of or an entire straw can inadvertently be left in the uterus. This can be the result of a prolonged and aggressive AI attempt by a novice, but can also occur when the subject is unruly and/or poorly restrained. These "foreign bodies" serve to function as excellent intrauterine devices and thus result in infertility. Depending on the duration of retention, it can be removed transcervically, which is best attempted during estrus, or surgically. Transcervical removal can be attempted with any long forceps, including an equine biopsy forceps. Often the straw becomes "embedded" in the endometrium. Removal in these cases can be facilitated by the use of a Foley catheter and the introduction of 80–150 ml of sterile lavage fluid. This serves to expand the uterus and help with removal. Surgical removal can be accomplished via a flank approach and I have utilized a colpotomy approach as well. In either case, if the straw appears intact it can simply be manipulated and forced out manually without an incision. If there are straw fragments, an incision must be made and the uterus lavaged following removal. Closure is with a disposable suture and pattern of individual preference.

Endometrial rupture during an ET flush is not uncommon and will occur following overextension of the Foley balloon, but also from unexpected and dramatic movement by the subject. In any case, it results in some level of hemorrhage, but also the inability to recover lavage fluid. Immediately stop flush attempts, administer 5–10 mg $PGF_{2\alpha}$ (Lutalyse®) intravenously via the tail vein, and decompress the Foley balloon. After 5–10 minutes have elapsed the flush can be resumed, this time replacing the catheter in a position anterior to the previous location.

Urovagina (Urine Pooling)

Urovagina is recognized as one of the most common reproductive disorders in the mare. Although there have been various reports that describe surgical correction of this condition in the cow [6–8], only relatively recently has work been published that describes risk factors, its effect on reproductive performance, and prevalence [9]. This work revealed that based on the evaluation of Holstein cows over a total of 344 lactations, this condition occurred at a prevalence of 26.7%. Urovagina was identified by vaginal speculum examination and classified as mild, moderate, or severe based on the amount of urine pooled within the vagina. Only moderate or severe categories of the condition resulted in a decrease in fertility and these in turn represented about half of the cases. Risk factors included poor body condition, a history of endometritis, and poor vulvar conformation.

This condition is therefore worthy of our attention and attempts at correction. Although risk factors have been identified, a specific etiology is less clear. A common case presentation at our referral clinic is that of urovagina in an aged donor cow with a likely iatrogenic etiology, specifically that being repeated treatment to induce superovulation. The reason I believe this is that most of the time these cows do not present with a torn or injured urethra or possess any of the identified risk factors, but instead a large vagina in which the cranial aspect falls over the floor of the pelvis. This condition of course can also occur following

injury, usually associated with dystocia, or is manifest as splanchtosis from multiple calvings. Perhaps because there is no single management or surgical procedure that is either easily performed or works in every case, several options will be discussed, the goal being to provide a comprehensive review and hopefully not that of either creating confusion or stimulating an argument on which is best.

One of the urethral extension procedures described for the mare can be utilized, although I have typically employed one of the easier alternatives. Regardless of etiology, if the case presented involves a cow to be used for ET and the client's desire is to simply get one more embryo flush, a long Foley catheter (20–24 French, 30-ml balloon) can be placed in the urethra at the initiation of the superovulatory regime and maintained through embryo flush/recovery. A one-way valve (finger of a latex glove with a small slit) is placed over the egress of the catheter to allow urine out but preclude air from entering. This will temporarily prevent urine from accumulating in the cranial vagina, but irritation to the bladder and urethra from the presence of the catheter limits its value as a long-term solution.

For a longer semipermanent correction, a circumferential suture placed in the vestibulovaginal junction (Figure 55.7) as described by Dr. González-Martín et al. [6] will allow several embryo recovery flushes or the maintenance of pregnancy (note that the suture should be removed prior to parturition). To accomplish this procedure the cow is restrained, epidural analgesia employed, the tail tied to the side, and the vagina and perineum prepared for surgery. Next, place a catheter into the bladder to prevent the inadvertent restriction of the urethra by the subsequent suture pattern. Introduce a hand into the vagina, utilizing either a hand-held curved cutting edge needle or a Deschamps needle (Figure 55.8) to place a subcutaneous suture cranial to the urethral orifice. The needle is introduced at the 4 o'clock position and exits at the 8 o'clock position. Then reintroduced at the same location (8 o'clock), exiting at the 12 o'clock position, and again reintroduce (same location – 12 o'clock), to finally exit at the original 4 o'clock location. Tighten the suture and tie. Note – a finger is placed in the lumen to provide a gauge for tightness of the cerclage. There should be ample opening for passage of an AI pipette or ET catheter. After several modification attempts of this approach the author has concluded that strict adherence to the procedure as described by González-Martín et al. will provide the best long-term results.

A technique described by Robert Hudson [10] that may offer permanent correction is the creation of a transverse dam across the floor of the vagina cranial to the urinary meatus, after which a Caslick procedure may be performed. The patient is prepared as with the other procedures, restrained, an epidural administered, and the vagina prepared with antiseptic lavage. Entering the vagina manually, a fold of tissue is grasped and rolled up at the level of the constrictor vestibuli muscle to create a transverse fold. Place an anchor suture with a half-curved, cutting-edge needle and either #3 or #4 catgut at the 4 o'clock position on the lateral wall, continuing with a mattress suture pattern in which you are alternately passing the needle cranially and caudally through the fold you created (Figure 55.9). Care should be taken to avoid the urethra and you should begin, proceed, and end the pattern keeping in mind the goal of incorporating 120° of the vaginal circumference and the creation of a semilunar dam of about 5 cm. Because of a "drawstring" effect that results from the mattress suture, there will not be a consistent height of the dam, but this does not affect the effectiveness. Adhesions should form within the tissues pulled together to form the dam, making this a permanent barrier to urine retention.

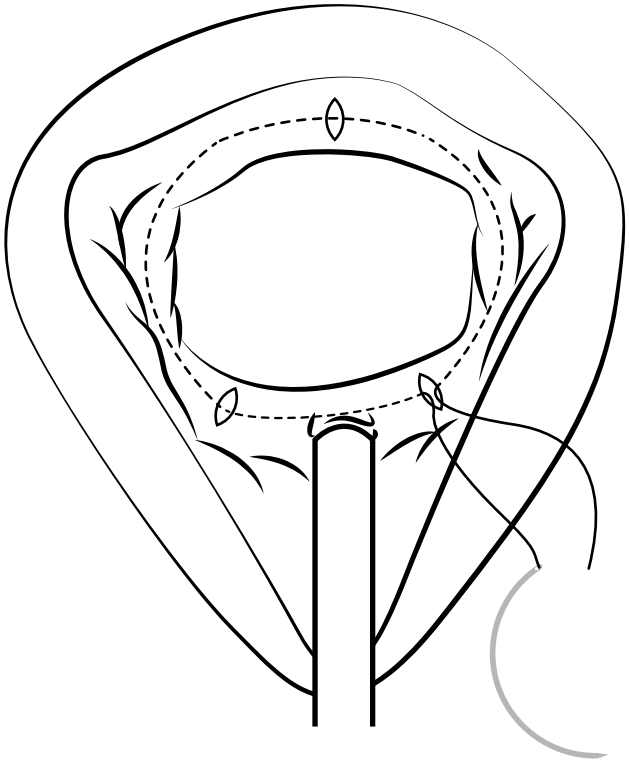


Figure 55.7 Suture placement for circumferential suture in the vestibule of the vagina. A catheter is placed in the urethra to avoid incorporating it into the suture. This procedure is described by González-Martín et al. [6]. *Source:* Image courtesy of McRae Hopper.

Additionally, the Minchev procedure or one of several of its modifications can be utilized. This is performed exactly as it is when utilized for vaginal or cervico-vaginal prolapse retention. It is effective for the resolution of urine pooling because it has the effect of both raising the vagina and pulling it caudally. This typically facilitates urine passage.



Figure 55.8 Deschamps needles. One of the instruments has been made longer. This may not be an improvement as this instrument is less stable and will bend under pressure.

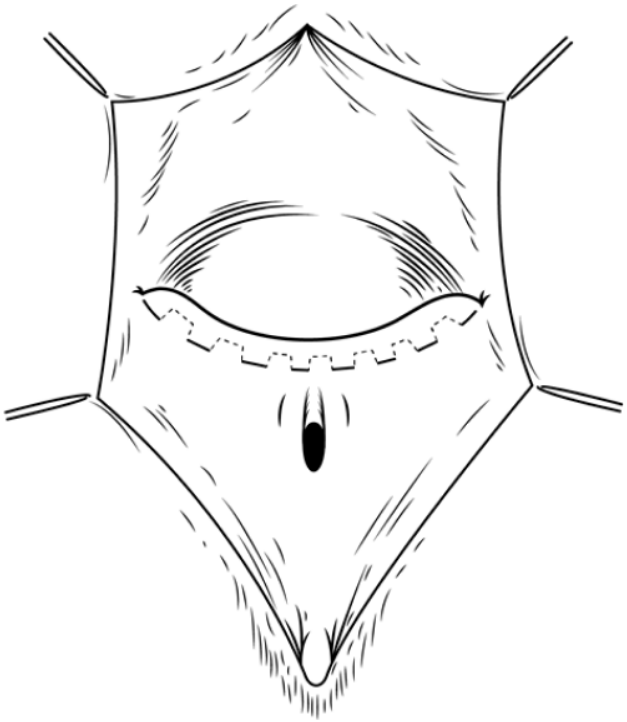


Figure 55.9 Transverse bridge formed by continuous mattress suture placed in skin fold cranial to urethral orifice. *Source:* Image courtesy of McRae Hopper. Adapted from [10].

Removal of a Mummified Fetus Via Colpotomy

A mummified fetus is an infrequent finding during routine herd pregnancy checks. Additionally, this condition is discovered when a cow is presented due to signs of anestrus or had been previously diagnosed pregnant and is overdue. Diagnosis can be made by per rectal palpation. An enlarged uterine horn with a palpably hard intrauterine mass, accompanied by a lack of fluid, is the most common finding, as well as the presence of a corpus luteum on the ipsilateral ovary. Utilization of ultrasonography is also of value (see Chapter 40).

Mummification of a fetus can occur any time after fetal ossification [11], typically between the third and eighth month of gestation. The condition is dependent on the maintenance of the corpus luteum and a closed cervix. Several etiologies for this condition are possible. Trichomoniasis and bovine viral diarrhea virus are the most common of the many infectious agents that cause early fetal death. Chromosomal abnormalities have been implicated as well as genetic anomalies. One genetic cause, an autosomal recessive gene for deficiency of uridine monophosphate synthesis (DUMPS), is known to cause embryonic and fetal death and was identified in the DNA extracted from two mummified fetuses [11].

Depending on the age of the cow and/or economic considerations, the cow may be immediately culled. If an attempt to treat the cow is chosen, conservative medical treatment should always be the first approach, this being the administration of a native or synthetic PG (Lutalyse®, Estrumate®) [12]. Depending on whether estrogen products are legally available for use in cattle in your country, a single dose of estrogen, either estradiol cypionate or estradiol benzoate (2–5 mg), can be given with or 24–48 hours after PG treatment with the goal of facilitating response by softening or "priming" the cervix. PGE available as a suppository used in human obstetrics, a compounded form, or Cytotec (misoprostol) tablets can also be used intracervically to aid in cervical dilatation. It is worthwhile to repeat medical treatment several times before resorting to either surgery or culling.

In cases in which there is no response to these treatments, a surgical remedy may be effective. A hysterotomy via either a flank approach or a colpotomy is recommended. In a retrospective study of cases not responsive to PG treatment [11], some mummified fetuses were removed by a combination of medical treatment (estradiol-17β, $PGF_{2\alpha}$, and oxytocin), followed by manual cervical dilation and manual extraction, and some by a flank approach hysterotomy. In this study 36% of cows became pregnant following a hysterotomy removal, while none conceived following the manual extraction.

A flank approach or a midline is recommended in cases in which the mummy is greater than 30 cm [13]. For those that are smaller, a hysterotomy via a colpotomy [14] approach can be performed easier and in less time. The cow is restrained in a chute. It is recommended but not absolutely

necessary to fast the cow, removing feed for 24 hours and water overnight. Sedation or tranquilization is dependent on the patient's personality. Administer an epidural and evacuate the rectum as effectively as possible. Then place a length of 7.5-cm stockinette packed with cotton within the rectum. Clean the vulva and prepare the vagina by lavaging with a dilute disinfectant (Betadine). Using dampened cotton pledgets, dry or at least remove water from the vaginal vault. Creating a pneumovagina by introducing or allowing air in will facilitate the procedure. With a blade carefully guarded between finger and thumb, introduce a hand into the vagina, and at a location in the anterior vagina that is dorsolateral (10 o'clock) to the cervix make a small stab incision. Alternatively, a specially made trocar can be utilized to make this stab incision. After an incision is made, the vagina is reentered and the incision is enlarged bluntly, first with the fingers and then the hand until a hand can be introduced into the abdomen. This should be performed aggressively and not gingerly, as overly cautious manual entrance serves to "tent" the peritoneum rather than push through it and this is actually more painful to the patient. Once the hand is inside the abdomen, the uterus is easily palpated; when the horn with the mummy is located, grasp and retract it out through the rent created in the vagina (Figures 55.10 and 55.11). Once it is exposed, make an incision into the uterus and extract the mummy. The lumen of the uterus is then lavaged to make sure that all foreign material is removed. Then suture the uterus with a continuous inverting suture pattern as you would a cesarean section hysterotomy. Rinse the uterus removing any debris or fibrin tags. Addition of carboxymethylcellulose to the saline used to both lavage the uterine lumen and cleanse the uterus before replacement is likely beneficial in reducing the possibility of adhesion formation. Then replace the uterus. Although suturing the rent created in the vagina is considered optional, I do not attempt to close colpotomy incisions on mares but I close it in cows. This can be done with a continuous pattern (the suture technique described for uterine tears can be utilized). If the decision is not to close the colpotomy incision, a Caslick should be performed.

Unilateral Ovariectomy

The indications for ovariectomy include neoplasia or the presence of severe adhesions, either of which affect the function of the ovary. There should be reasonable evidence that the contralateral ovary is normal and that fertility will be restored following removal of the abnormal ovary. An ovary that is small enough to be easily grasped during per rectal palpation can be removed via colpotomy. Larger ovaries or those with extensive adhesions should be removed via flank celiotomy.



Figure 55.10 The uterus (uterine horn containing a mummified fetus) exposed through a colpotomy incision.



Figure 55.11 Incision into the exposed uterine horn.

Ovariectomy Via Colpotomy

The colpotomy approach was described in a previous section. Once the abdomen has been entered, the chain loop of an écraseur is placed around the ovary. The chain loop is slowly tightened, making sure that the placement does not include the bowel. The loop is tightened further until the ovary's attachment is severed and the ovary can be removed. Although it is recommended that only smaller ovaries (those that can easily be held in the operator's hand) are to be removed by this technique, larger ovaries can be removed by carrying a sterile plastic bag into the abdomen, dropping the ovary into the bag, and then removing the bag with the ovary.

Ovariectomy Via Celiotomy

The left flank celiotomy approach is described in Chapter 52 and the only difference is that the incision for this should

be made slightly more dorsal and caudal. This approach is preferred over the colpotomy when the ovary to be removed is large, adhesions are severe, or there is concern relating to hemostasis.

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Pregnancy Wastage

56

Fetal Disease and Abortion: Diagnosis and Causes

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Introduction

Reproductive failure is a significant problem in breeding management that arises from physiological, anatomical, inherited, and infectious causes. The inability to achieve or maintain a pregnancy may be divided into four main stages: failure to ovulate after estrus, failure of fertilization, embryonic death (prior to gestation day 42), and fetal death [1]. Pregnancy wastage, which probably comprises the largest portion of these losses, encompasses the combined embryonic, fetal, and neonatal deaths that achieve nothing. It has been estimated that about 75% of pregnancy wastage occurs in the embryonic stage [2, 3]. The majority of reproductive failure occurs in the first two weeks, at the time of development from a morula to blastocyst where the conceptus begins enhanced protein synthesis and placental development [3–6]. Despite the importance of wastage at this time of development, the mechanisms contributing to embryonic death are poorly understood and in most cases the cause is never established [1]. Factors typically thought to contribute to embryonic death include heat stress, infections of the uterus/gametes/embryo, local trauma, genetic factors, heavy lactation in dairy cattle causing energy imbalances, maternal illness, aged oocytes from persistent follicles, small follicles, fetal–maternal incompatibilities, twinning, postpartum breeding intervals, and abnormal progesterone levels due to estrus synchronization [1, 3, 6, 7]. Investigation into the causes of early loss are hampered by the rarity of available early conceptuses that are either expelled or resorbed and go unnoticed, only to result in a cow that returns to estrus or fails to deliver a calf.

Abortion typically refers to pregnancy loss in the fetal stage, between days 42 and 260. It is in this stage of gestation that the tissues of conception cannot be resorbed and, when expelled, are more easily noticed. During the fetal period there is a progressive decrease over time in the risk of abortion, with a slight increase in the last month [8]. Although losses during this time are a minority of overall wastage, the cost of investment by the cow and the manager is substantial. A mid-term abortion represents a loss of at least USD $600–1000 [8]. For practical purposes, unavoidable losses of 3% after pregnancy confirmation (six weeks' gestation), with 1–2% loss in the periparturient period, may be considered acceptable or typical, although opinion in this area varies [8–10].

This discussion will concentrate on fetal death and the approach to diagnosis, but will necessarily include some aspects of embryonic death, congenital abnormalities, and common gross findings. Additionally, a brief overview of anatomy and development are included to familiarize the reader with general concepts. A glossary of terms appears at the end of this chapter.

The investigation of abortion is a vital part of herd management and its importance is hard to overemphasize. Accurate diagnosis is the only path to the effective control of disease. It should go without saying that a focused, efficient, and thorough investigation technique with a definite purpose by prepared veterinarians is the only relevant way to identify and understand the etiologies of abortion. The necropsy itself contributes to this in several ways: by establishing definitive causes of death, identifying unsuspected findings, providing information concerning zoonotic disease, contributing to discovering new diseases and pathogenic mechanisms, and providing a means to test new diagnostic and treatment techniques [11].

Pathology

The expression of disease in the conceptus is remarkably varied in a general sense, in that it gives rise to bizarre developmental defects of potentially every sort. This is due

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to the intricate physiology and morphology of fertilization and gestation that is shared between the dam and fetus. The sequential stages of organ system development in utero provide unique opportunities for infectious and non-infectious etiologies to manifest themselves at the gross level, if pregnancy is maintained. In addition, the placenta, a unique organ of gestational necessity, is a target of many disease-causing agents and can itself develop anomalous defects that in turn directly affect the fetus.

Despite this, in many cases, aborted tissues from infectious and non-infectious causes exhibit little if any recognizable changes of significance. This is partly due to autolysis in utero masking subtle changes; the relative ease and rapidity by which fetuses succumb to disease, allowing only a brief window for gross changes to manifest; and a rudimentary inflammatory response to injury [12]. Not only does the conceptus vary in its susceptibility to particular insults across the gestational period, but also the degree and duration of insult determine the outcome, whether it be life, death, malformation, or inflammation [9].

The physiology of pregnancy is well studied and dealt with in detail in preceding chapters. Despite our understanding of normal pregnancy, the physiology of abortion and the mechanisms involved are poorly understood. In many cases, abortion may be mediated through the same pathways that occur in normal parturition, but different mechanisms and pathways are also possible [8].

Categories of Reproductive Loss

The cause of abortion is often not known, which is a consistent source of frustration for managers and clinicians alike. In all species, abortion may be caused by infectious and non-infectious etiologies. Specific causes and agents are dealt with in detail later in this and subsequent chapters. Of these, infectious causes of abortion (bacteria, viruses, fungi, protozoa) are probably the best understood and characterized. It is common to see reports where the percentage of cases with a specific diagnosis is less than 50% [13]. Of these, at least half are due to infectious agents, with the majority of these due to bacterial infection [8].

Bacteria involved in abortions can be broadly grouped into those that are contagious and those that are opportunistic. The majority of bacterial abortions are caused by opportunists, and these may be further divided into those that are part of the natural flora, such as *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) and *Histophilus somni*, and those from the environment, such as *Bacillus* spp. and *Escherichia coli* [14]. The significance attached to abortion by opportunists depends on the situation in the herd. If these bacteria are only seen in isolated cases, the significance to the herd is minimal [14]. However, if these organisms are consistently associated with abortions, then further investigation is warranted.

Table 56.1 Disease-causing agents associated with endemic fetal losses.

Histophilus somni, *Listeria monocytogenes*, *Trueperella pyogenes*, *Leptospira* spp., *Ureaplasma diversum*
Bluetongue virus, bovine viral diarrhea virus (BVDV)
Neospora caninum, fungi, epizootic bovine abortion
Inbreeding, sire-derived lethal traits, chromosomal abnormalities
Feed estrogens (silage, poultry litter), progesterone aberrations (high pasture protein)
Protein, vitamin A, iodine, selenium deficiency
Protein/urea, copper, iodine excess
Endotoxins due to Gram-negative bacterial sepsis in dam
Endotoxin in Gram-negative bacterial vaccines, especially given during the first or last two months
Pine needle, broomweed, locoweed, narrow leaf sumpweed toxicosis
High plant estrogens
Aflatoxin, ergotamine, fusarium (zearalenone), nitrate fertilizer, organophosphate toxicosis

Source: [15].

Non-infectious causes of abortion are similar to those known to cause embryonic loss, including nutritional imbalances, malnutrition, stress, environmental toxins, teratogenic compounds, hormone imbalances, and genetic abnormalities. An expanded list of etiologic agents is shown in Table 56.1 and in the following chapters.

Pathophysiology of Injury to the Conceptus

Maintenance of the first half of gestation (the first 200 days) in cattle requires a persistent corpus luteum (CL), which is maintained by the fetus [9, 16]. Luteolysis may occur in pregnant cows due to excess prostaglandins (PGs) (exogenous administration or secondary to heat stress) or Gram-negative bacterial septicemia [17]. If the CL is destroyed at an early stage, death and rapid loss of the embryo with minimal degeneration may be seen [18]. If there is fetal death prior to luteolysis, the CL may regress, with eventual expulsion of autolyzed fetal tissues. However, in some cases the CL is maintained after fetal death, which can result in expulsion, resorption, or mummification [19]. Early abortions are typically not recognized; most are occultly expelled, severely autolyzed, or mummified.

The maintenance of late-stage pregnancy requires both the fetus and placenta. At this stage in development, sufficient fetal stress can induce parturition through natural endocrine mechanisms. In this way, chronically diseased fetuses can initiate their own premature delivery.

Fetal death often leads to abortion within a few days rather than immediately, allowing sufficient time for autolytic changes to occur. The mechanism for expulsion of dead fetuses is unknown but may share many similarities with normal parturition [19]. Autolytic changes include generalized edema, pallor, and hemoglobin-stained fluids in the body cavities. Visceral tissues become pulpy or semiliquid [19]. In some cases, the time between fetal death and expulsion may be characteristic for a pathogen, and may therefore be useful to the clinician. Fetal infections due to *Listeria monocytogenes*, *T. pyogenes*, non-septate fungi (*Absidia* spp., *Mucor* spp., *Rhizopus* spp.), and bovine herpesvirus (BHV)-1 may lead to fetal death with expulsion days later. Fetuses infected by *Campylobacter fetus* and *Aspergillus* spp. may be delivered alive [20].

It is important to recognize that infection of the conceptus does not necessarily lead to fetal death. The ability of a pathogen to injure the conceptus is influenced by the dam (general health, previous exposure), the stage of fetal development, and the virulence of the infectious agent. Fetal development is a continuum of organogenesis, physiological development, and immune development. Early stages of gestation are more prone to infection and severe disease, as the capabilities of the fetus are underdeveloped. The closer the fetus is to parturition, the stronger and more capable it is of defending itself from pathogens. Thus, infection at different stages of development will produce different outcomes in the fetus.

Bovine viral diarrhea virus (BVDV) infection is a good example of this. Infection in the first trimester often leads to fetal death and resorption, while infection in the second trimester may lead to developmental anomalies. The character of the developmental defects depends on which cells or tissues are susceptible to the virus at the time of infection. In the second trimester, cerebellar growth is maximal and BVDV infection at this time may lead to cerebellar necrosis and cerebellar hypoplasia. Also at this time, hair growth is highly active and BVDV infection may damage follicles, resulting in hypotrichia. By the third trimester, the immunocompetent fetus may react to BVDV infection by mounting a sufficient immune response with antibody production and virus elimination. The only evidence for such an infection would be fetal-specific antibody titers [21].

Other viruses, such as BHV-1, cause extensive cell necrosis and hemorrhage, leading to rapid fetal death. *Neospora caninum* infection in the third trimester may cause only mild inflammation, with the birth of asymptomatic calves.

Effects of Maternal Disease

Infectious pathogens may cause abortion either by directly infecting the conceptus or by affecting the dam such that severe physiological disturbance leads to abortion. Uterine disease has significant effects on the conceptus and potential future fertility; ovarian and uterine effects can be lasting despite resolution of the signs of active disease [22]. The dam is predisposed to infection through non-specific immunosuppression that occurs during pregnancy [23]. Fever (mastitis, pneumonia), circulatory disease (myocarditis, severe anemia), hypoxia, and endotoxemia (Gram-negative sepsis) are possible conditions that may cause abortion indirectly [21]. PGs may be elevated in febrile states, which can lead to luteolysis and abortion [24]. The likelihood of abortion probably increases with the number of pregnancies or previous abortions by the dam [8].

Routes of Infection to the Conceptus

In cases where infectious organisms invade the conceptus, four routes are likely [21]. Hematogenous spread from the dam to the placenta is commonly suspected in cases of infectious abortion, particularly due to *L. monocytogenes*, *Leptospira interrogans*, *Salmonella enterica*, *Brucella abortus*, fungus, BHV-1, or BVDV infection. Ascending infection from the vagina through the cervix can occur from primary vaginitis (characteristic of *Tritrichomonas foetus*) or contamination at insemination. The presence of pathogens within the uterus (endometritis due to *C. fetus*, *T. pyogenes*) and descent from the abdomen via the uterine tubes (mycobacteriosis) are also possible pathways for organisms to reach the conceptus.

Route from the Placenta to the Fetus

Once a pathogen reaches the placenta, it may proceed to the fetus through the blood, via the umbilical veins, or by contamination of the amniotic fluid [21] (Figure 56.1). For example, BVDV primarily infects the fetus hematogenously, whereas BHV-1 may first infect the placenta and then proceed to the fetus. Primary placental infection with subsequent amniotic fluid contamination is characteristic of fungal infections. In some cases, a pathogen need not infect the fetus, such as with *S. enterica* infection, where severe infection of the placenta may lead to generalized fetal hypoxia and subsequent death.

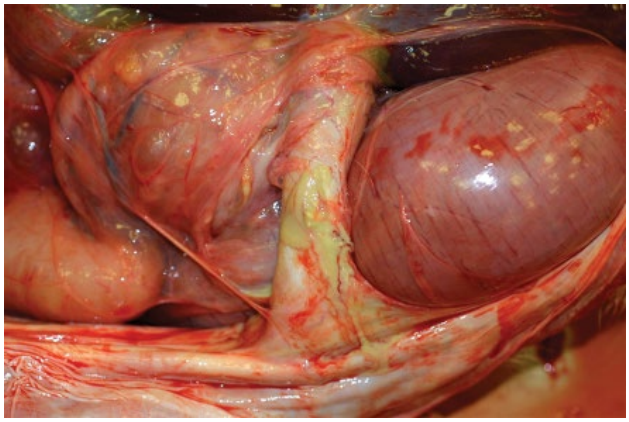


Figure 56.1 Fetus, omphalitis, and ascending bacterial infection from the placenta. Purulent material is easily expressed from the cord. *Source:* Photo courtesy of J. Edwards.

Lesions of the Placenta

Placentitis most commonly develops by one of three ways: hematogenous spread, extension from a diseased uterus, or an ascending vaginal infection. Gross placental lesions are characteristic of chronic bacterial and fungal disease, while viral infections generally produce no lesions [25]. Changes of significance may occur in both the cotyledons and/or intercotyledonary spaces. The presence of fibrin (yellow, stringy, friable material) on cotyledons is an indication of inflammation (Figure 56.2). Fibrin must be distinguished from necrotic inflammatory exudate or entrapped caruncle fragments (necrotic uninfected endometrial tissue). The presence of ecchymosis and extension of the changes to the intercotyledonary space are suggestive of an infectious inflammatory process. With chronicity, stereotypical changes of placentitis include (i) placental fibrosis and edema; (ii) cupping of cotyledons; (iii) exudate on chorionic surfaces; and (iv) necrosis of the cotyledons [12].

Fungal and bacterial inflammation of the cotyledons is often associated with necrosis, which imparts a firm to friable, tan, irregular or nodular consistency to the tissues with interspersed ecchymoses. Fungal organisms prefer to grow along and within blood vessels; this often leads to thrombosis, hemorrhage, and necrosis (Figure 56.3). Fungal organisms may be particularly abundant at the periphery of lesions rather than in the centers. A useful feature for interpretation is the extension of the pathologic process into the pericotyledonary chorioallantois. Such infiltrates may bridge cotyledons and often impart a leathery thickened texture. Another differential for such a change would be adventitial placentation (discussed in the section "Pathology"). Leptospirosis and BHV-1 infections may also produce a placentitis and appear similar to one another [26].

Ureaplasma diversum is known to produce a rather characteristic hemorrhagic amnionitis, where the membranes

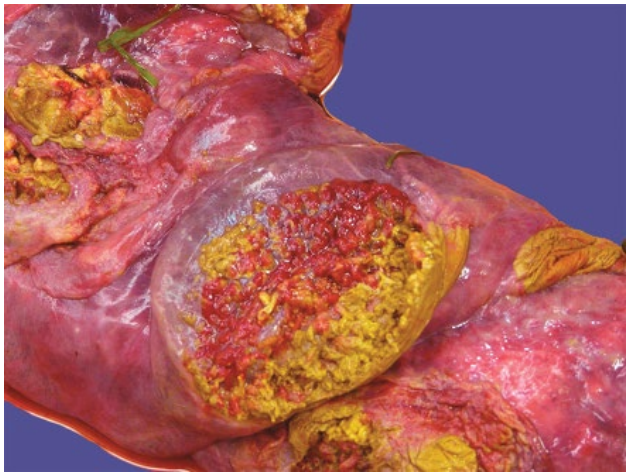


Figure 56.2 Chorioallantois and placentitis. The cotyledons are thickened with yellow discoloration due to fibrin and necrosis. Intercotyledonary tissues are relatively unaffected. *Source:* Photo courtesy of J. Cooley.

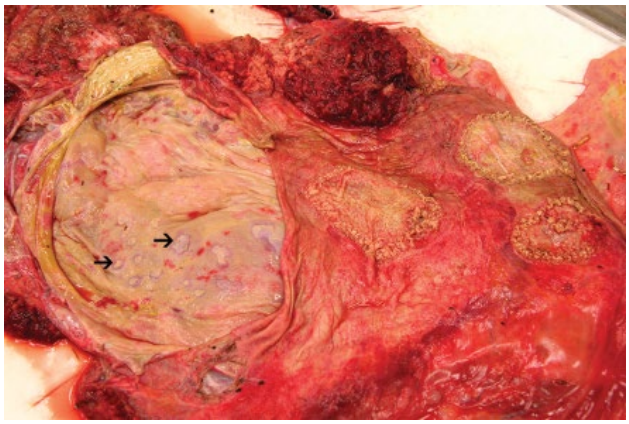


Figure 56.3 Chorioallantois and fungal placentitis; maternal aspect on right, fetal aspect on left. Cotyledons are thick, irregular, and tan to hemorrhagic. Intercotyledonary areas are thickened. The fetal side shows yellow discoloration and multiple discrete infarcts (arrows) due to vascular thrombosis. *Source:* Photo courtesy of J. Edwards.

are thickened, opaque, and ecchymotic with fibrin, necrosis, and fibrosis [27].

Lesions of the Fetus

Gross lesions of the fetus, aside from malformations, are generally uncommonly noticed. They may be inapparent for four reasons: (i) the fetal immune system is not of sufficient robustness to mount a response that is easily observed at the gross level; (ii) fetuses often die before lesions appear; (iii) lesions are masked by autolysis; and (iv) fetal pathology may manifest itself in ways that are difficult to recognize [15]. Table 56.2 lists some causes for commonly

Table 56.2 Possible causes for gross and microscopic lesions in bovine fetuses.

Lesion	Possible causes
Gross lesion	
Mummification	BVDV, *Neospora*
Ascites/anasarca	Congenital heart defect, nutritional myopathy/cardiac dilation, BVDV, *Neospora*
Arthrogryposis, musculoskeletal deformities	Akabane/Schmallenberg/bunyaviruses, reduced in utero motility, genetic
Fibrinous peritonitis/pleuritis	Bacteria (*Trueperella*, *Campylobacter*, *Bacillus*, *Brucella*, *Chlamydia*), fungi, EBA
Fibrinous pericarditis	*Bacillus*, *Campylobacter*
Icterus	*Leptospira*
Cerebellar hypoplasia	BVDV, Schmallenberg, BTV
Hydrocephalus, hydranencephaly, porencephaly	BTV, BVDV, SBV, other bunyaviruses, genetic anomaly, toxins (lupines), genetic
Microphthalmia	BVDV
Pulmonary/renal hypoplasia	BVDV
Dermatitis/hyperkeratosis	Fungi, EBA
Multifocal liver necrosis	*Listeria*, BHV-1, *Yersinia*, *Salmonella*
Splenomegaly/lymphadenopathy	EBA, HoBi-like pestivirus
Hepatomegaly	Chronic passive congestion, EBA, lipodystrophy
Placentitis, infarctions	Fungi
Placental exudate	Bacteria (*Trueperella*, *Campylobacter*, *Brucella*, *Leptospira*, Mollicutes, *Coxiella*), *Trichomonas*, BHV-1
Generalized petechiation/hemorrhage	BVDV, anthrax, HoBi-like pestivirus
Microscopic lesion	
Encephalitis	*Neospora*, BHV-1, BVDV
Meningitis	EBA, *Leptospira*, *Brucella*, other bacteria
Myocarditis	*Neospora*, BVDV
Suppurative bronchopneumonia	Bacteria
Bronchointerstitial pneumonia	*Ureaplasma*, *Brucella*
Multifocal hepatic necrosis	BHV-1, *Listeria*, *Salmonella*, *Yersinia*
Interstitial nephritis	*Neospora*, *Leptospira*
Abomasitis/enterocolitis	Bacteria, fungi
Conjunctivitis	Bacteria, *Ureaplasma*, fungi
Placentitis	Bacteria, fungi
Oxalate crystals, renal	Congenital metabolic defect, associated with anomalies, ingestion of oxalate-producing plants by dam
Liver: (1) Lipid; (2) fibrosis; (3) iron	(1) Lipid-toxin exposure, lipodystrophy; (2) fibrosis-aflatoxin, passive congestion; (3) iron-weak calf syndrome

Source: Based on [28].
BVDV, Bovine viral diarrhea virus; BTV, bluetongue virus; EBA, epizootic bovine abortion; BHV-1, bovine herpesvirus 1; SBV, Schmallenberg bunyavirus.

encountered lesions in aborted fetuses. Other conditions are listed further in the text.

The most common finding in abortion is autolysis, the degree of which depends on the cause of death, the time from death to abortion, and the time from abortion to examination [26]. In experimentally induced, sterile, dead ovine fetuses, changes associated strictly with autolysis included (i) lack of odor, (ii) subcutaneous blood-tinged gelatinous edema, (iii) blood-tinged fluids in body cavities, (iv) renal cortex softening, (v) liver softening, (vi) abomasal content that was cloudy yellow to red, and (vii) uniform color (pink/red) of tissues [29] (Figures 56.4 and 56.5). By

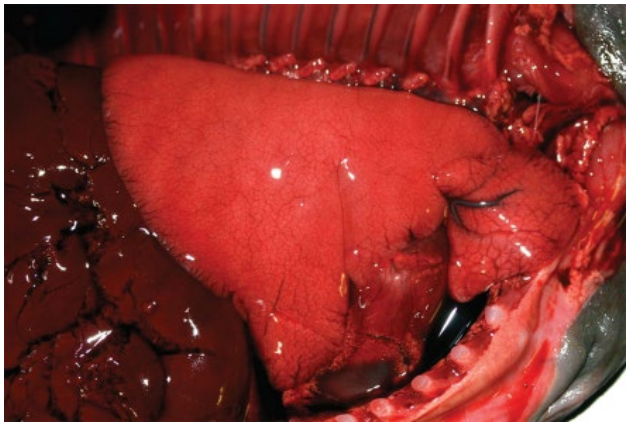


Figure 56.4 Aborted fetus; opened chest and abdomen. The lungs are uninflated (congenital atelectasis) and red with a shiny smooth pleura. Autolytic change is present (generalized reddening of tissues, friable liver). *Source:* Photo courtesy of J. Edwards.



Figure 56.5 Fetus and placenta. The fetus exhibits generalized red discoloration due to autolysis. *Source:* Photo courtesy of J. Cooley.

12 hours after death, the fetal corneas were cloudy, the liver and kidneys were friable, and abomasal content became cloudy with brown flecks. After 36 hours, the subcutis contained edema and the skin would slough. By 144 hours, progressive dehydration was obvious (mummification) [29]. Signs of fetal infection include fibrinous exudates in body cavities, white to tan foci in the liver or lungs, and evidence of abnormal development [26] (Figure 56.6).

Pathogens that invade the fetus via umbilical veins may produce lesions in the liver, as it is the first organ encountered (Figure 56.7). *L. monocytogenes*, BHV-1, *Yersinia pseudotuberculosis*, and *S. enterica* infections are often associated with liver necrosis. For pathogens that infect the amniotic fluid, exposure of the skin, lung, and intestines may occur. Skin lesions are particularly well known in cases of fungal infection, where pale patches of thickened skin are evident (Figure 56.8). Lung and pleural disease may be manifestations of hematogenous systemic spread or inhalation of infected amniotic fluid during fetal distress [21].

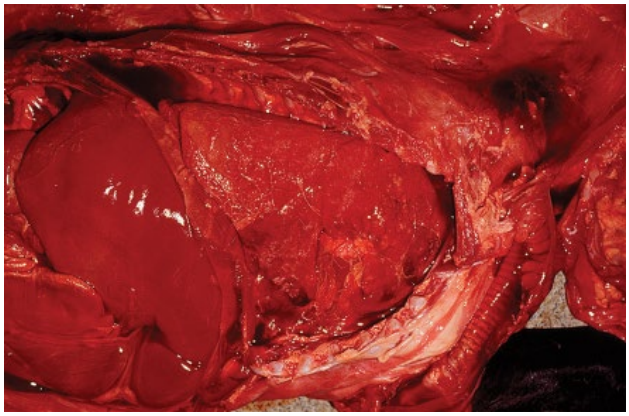


Figure 56.6 Aborted fetus with *Trueperella pyogenes* infection; opened chest and abdomen. Fibrin strands are present on the pleural surfaces. Autolytic change (generalized reddening of tissues) is evident. *Source:* Photo courtesy of J. Cooley.



Figure 56.7 Fetal liver section with hepatitis. Multiple tan foci of necrosis are surrounded by hyperemic parenchyma. *Source:* Photo courtesy of J. Cooley.



Figure 56.8 Fetus with fungal dermatitis. Coalescing, mildly bulging, gray/tan foci are present in the skin of the neck. *Source:* Photo courtesy of J. Edwards.

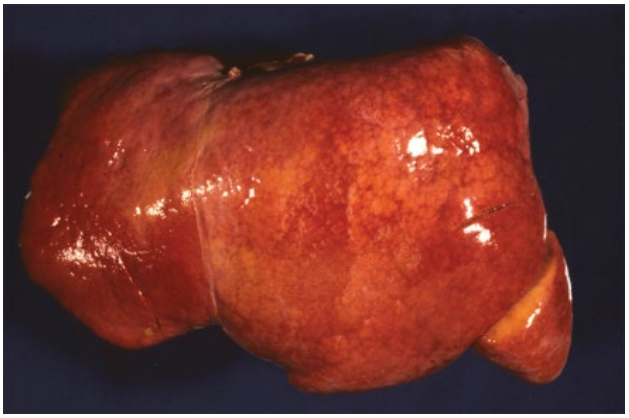


Figure 56.9 Fetal liver. The surface is tan and coarsely irregular. This change may be seen with chronic passive congestion (heart anomalies) or epizootic bovine abortion. *Source:* Photo courtesy of J. Edwards.

Epizootic bovine abortion (EBA) causes distinctive lesions in the fetal liver, lymph nodes, and spleen. The liver is irregular and nodular; however, this change may also be seen in cases of congenital heart disease where chronic passive congestion occurs (Figure 56.9). The spleen and lymph nodes are characteristically enlarged due to lymphoid and mononuclear cell hyperplasia [19]. Yellow/fatty, enlarged, and even fibrotic/cirrhotic livers may be seen in hepatic lipodystrophy, a poorly understood condition that may be related to selenium deficiency, toxin exposure (pyrrolizidine alkaloid, aflatoxin), or other genetic causes [30, 31]. Iron in the liver was reported in association with weak calf syndrome (possibly due to selenium deficiency) [32].

Outcome of Exposure to Abortifacient Agents

Fetuses exposed to abortifacient agents or conditions may die (resorption/abortion/stillbirth), they may be infected (with or without disease), they may be malformed (live/dead), or they may be normal [23].

Death in the embryonic stage leads to resorption. In the fetal stage the presence of skin and musculoskeletal structures, in addition to placental endocrine activity, prevents resorption. Instead fetal death leads to autolysis and expulsion, or retention with mummification/maceration. In late gestation, a sick or stressed fetus may precipitate its own premature delivery with one of three results: the fetus may be born and then die, it may be born and fail to thrive, or it may be born and thrive even if infected [21].

Disease may impair fetal motion, a necessary requirement for proper musculoskeletal development. In such cases, the calves may exhibit severe changes, including arthrogryposis, ankylosis, scoliosis, torticollis, and similar defects that lead to dystocia, stillbirth, or death in the perinatal period.

Teratology

Abnormal development (congenital defects) in the fetus may be caused by teratogens: toxic substances, infectious disease agents (particularly viruses), nutritional imbalances, endocrine imbalances, hypoxia, extreme temperatures, and inherited or spontaneous genetic mutations [33, 34]. The manifestation of these defects includes resorption/abortion, malformation, alteration of growth, and functional deficits (Figure 56.10). The late embryonic period and early fetal periods are the times of greatest susceptibility for the conceptus. During this time, each organ system is established in an orderly fashion with morphogenesis occurring in "critical periods." During these periods organs are at their most sensitive to teratogens.

In many cases congenital defects have no defined cause. Seasonal occurrence, history of stress, or maternal disease are often noted, with or without a familial component [35]. The genetic composition of the fetus as well as the nature and degree of insult figure largely in the outcome [13]. Genetic considerations are discussed in greater detail in Chapter 70.

In cattle, viral infections are well-known causes of defects. BVDV, infectious bovine rhinotracheitis (IBR), (BHV-1), Wesselsbron, bluetongue, Akabane (bunyaviruses including Schmallenberg), and Rift Valley fever (RVF) viruses are commonly associated with embryonic loss and neuromuscular defects [36, 37]. In addition, toxic plants such as lupines, *Conium maculatum*, and locoweeds are associated with defects [35]. Further discussion of such toxins is presented in Chapter 69.

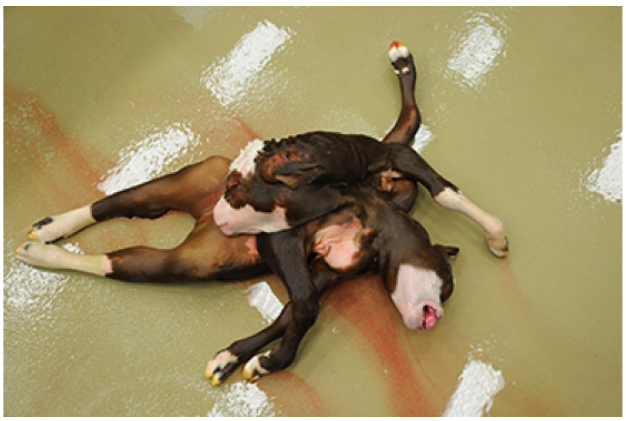


Figure 56.10 Fetal monster. A thoracopagus with shared viscera and four forelimbs.

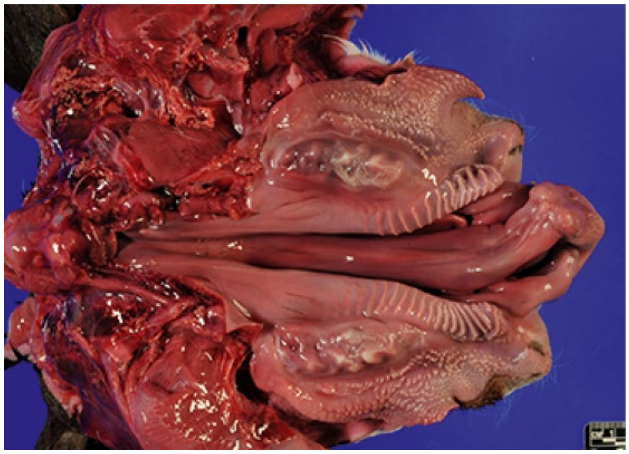


Figure 56.11 Palatocheiloschisis; cleft palate and lip (mandible removed).

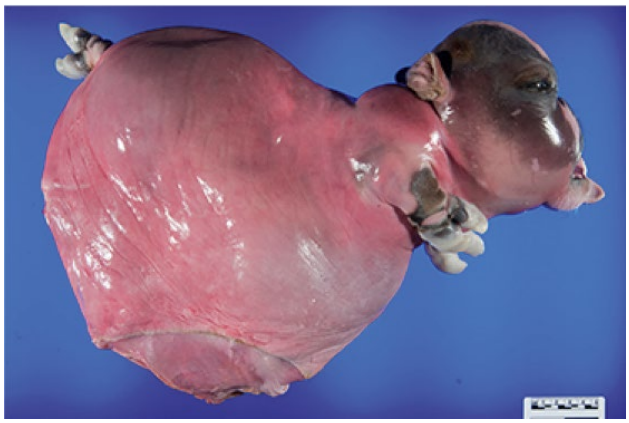


Figure 56.12 Chondrodysplasia; Dexter "bulldog" calf.

Calves with congenital defects often die during parturition or soon thereafter. In many cases the anomalies are grossly obvious, often affecting the neural and musculoskeletal systems (Figure 56.11). A wide range of malformations have been described, some of which have been intensively studied and a genetic component has been found (Figure 56.12). For the purposes of this chapter, relevant anomalies are generally discussed for those that are caused by infectious agents (BVDV, Akabane virus) or those that are not known to be inherited but are particularly puzzling when encountered (acardiac twins, moles). Reviews are available [13].

Neoplasia

Congenital tumors and neoplasms are rare in calves and are most often diagnosed as lymphomas, mesotheliomas, embryonic tumors, and hamartomas. They have been reviewed [31].

Anatomy

(See Figure 56.13 for identification of some anatomical terms.) The placenta is a transient metabolic organ derived from the fetal chorion and maternal endometrium to provide nutrition and metabolic exchange between mother and calf. Gestation can be divided into the embryonic stage with organogenesis and the fetal stage with fetal growth. The embryonic stage extends to around day 45; the fetal stage continues until delivery. Together, the chorioallantois and amnion form the extraembryonic fetal membranes.

After fertilization, the zygote undergoes cleavage to form a ball of cells (morula). With further division the morula develops into the blastocyst, which is an inner cell mass (the embryo proper) and a blastocoele (fluid-filled cavity) lined by the trophoblast cell layer. The blastocyst, now about 1.5 mm wide, hatches from the zona pellucida at 9–11 days after ovulation in order to attach to the endometrium of the uterus [38]. By 19 days, the bovine blastocyst trophoblast enlarges as a threadlike emanation/sac along the entire length of the uterine horn [37, 39]. At this time, defective embryos will die and be resorbed or expelled. Also, the CL is developing and producing progesterone [13].

The primitive endoderm from the inner cell mass migrates along the inner aspect of the trophoblast, lining the cavity that will become the yolk sac. The yolk sac provides nourishment to the conceptus and has developed on about day 20, but will quickly regress to an inapparent rudiment by day 30 [39–41]. At this time, prior to uterine attachment, the conceptus derives nourishment from the secretions of the uterine mucosal glands, known as histotrophe. This material is a yellow/white, opaque, thick secretion sometimes mistaken for purulent exudate [13]. Once the fetomaternal placental circulation develops, fetal nutrition is supplied by diffusion of nutrients from the blood across the placentome (hemotrophe) [37].

The primitive mesoderm migrates between the endoderm and trophoblast (now a trophectoderm), fusing with the latter to form the chorion (somatopleure). The chorion then rapidly expands as a transparent sac to fill the uterine lumen [42]. Around this time, dorsomedially migrating folds composed of mesoderm/trophectoderm surround the embryo between days 13 and 16, providing complete envelopment of the embryo in an amniotic cavity (vesicle) by day 35 [43]. The site where these folds meet, the raphe or mesamnion, persists as a broad attachment of the dorsum of the amnion to the chorioallantois in Bovidae. The amnion is therefore composed of an inner trophectoderm and subjacent mesoderm with extensive attachment to the allantois.

Between days 14 and 21, a diverticulum of the hindgut extends from the embryo into the mesoderm of the umbilicus to form the allantois (allantoic vesicle) and, along with

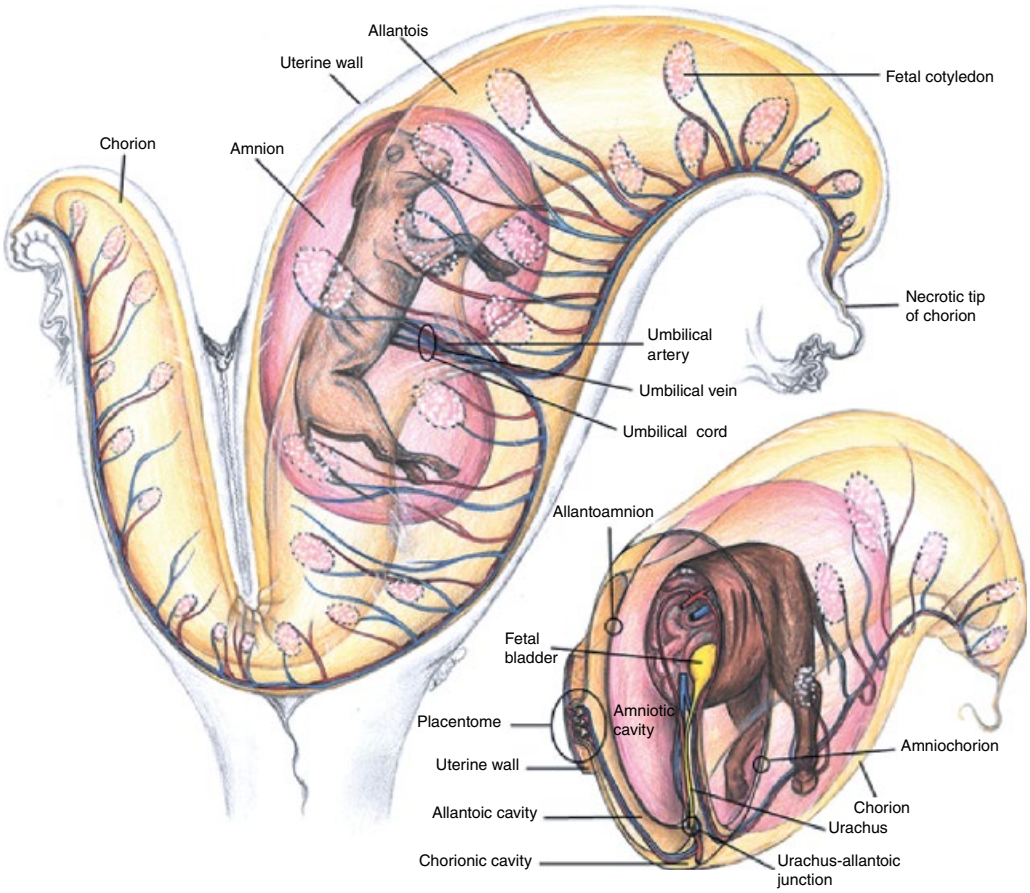


Figure 56.13 Schematic representations of the fetus in-situ during mid-gestation. *Source:* Images redrawn by Kirstin Cook from K. June Mullins and Richard Saacke *Illustrated Anatomy of the Bovine Male and Female Reproductive Tract*. Germinal Dimensions Inc., Cadmus Professional Communications, Science Press Division, Ephrata, PA, 2003.

it, the vasculature supplying the chorion and amnion [25]. The allantois continues to expand within the exocoelom to fill both uterine horns, with widespread apposition and eventual fusion to the chorion (chorioallantois). It is thus T-shaped, with the stem of the T as the allantoic stalk of the umbilicus and the other ends of the T as extensions of the chorioallantois into both uterine horns [37]. The chorioallantois attaches at four weeks' gestation to the uterus, forming irregular villous projections over the uterine caruncles, with eventual development into placentomes.

By days 30–35, three to four fragile attachments (early placentomes) are present in the pregnant horn; by day 40 a maximum of 20 attachments are present in both horns. At day 70, 80–90 placentomes are present, which will number 75–140 by mid gestation. Placentome size may vary widely, from 5 to 15 cm in diameter. The fetal cotyledon has a velvety red surface (Figures 56.14 and 56.15). Both the weight and size of placentomes increase during gestation, particularly in the pregnant horn [44]. Between placentomes, there is minimal chorioallantoic villous proliferation, which may be seen as fine red velvety spots occurring diffusely or in multiple, 5- to 10-mm-wide, regularly spaced foci. Such proliferation may cover non-gravid horns that contain no cotyledons.

Placentomes are usually arranged in two dorsal and two ventral rows along both horns, although an extra row or reduced rows may be seen (see Figure 56.13). They become progressively larger the closer they are to the fetus and the majority of cotyledons are present in the gravid horn. The largest placentomes are closest to the main arteries and become progressively smaller toward the periphery. In some cases all cotyledons are present in the gravid horn with few to no cotyledons on the non-gravid side. The cotyledonary rows may be interrupted by a circular bare area associated with the junction of the horns, near the cervix [45].

As development progresses, there is fusion of membranes to one another in the areas where they come into contact (see Figure 56.13). These attachments may be transient, but fusion of the allantois and chorion occurs around days 50–60 [43]. In early gestation, the amnion is relatively large and compresses the allantois laterally. As the amnion

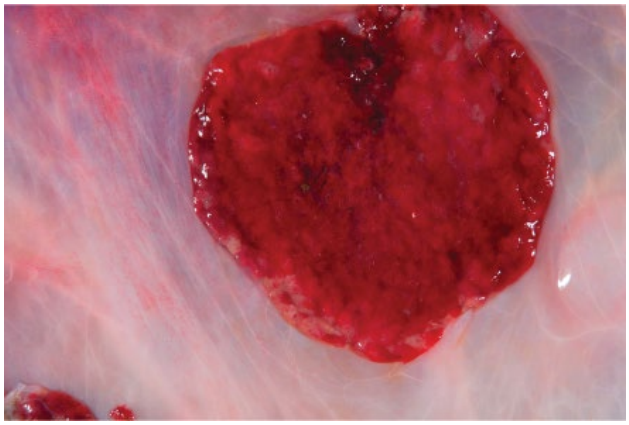


Figure 56.14 Normal placenta. Cotyledon has a red velvety surface. The intercotyledonary spaces are smooth, shiny, opaque, and white.

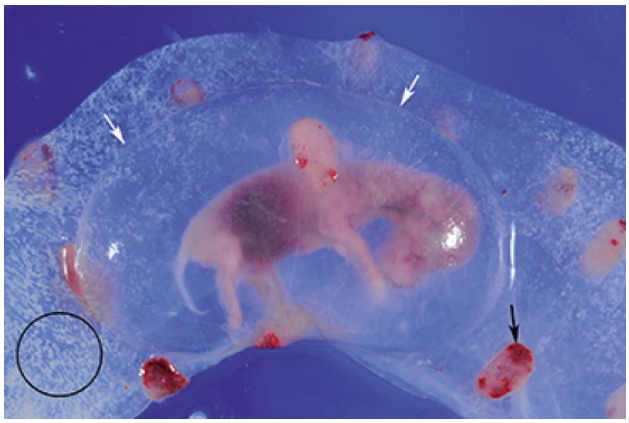


Figure 56.15 Normal fetus and placenta after 2 months' gestation. The calf is suspended in the amnion (white arrows), which is further surrounded by the chorioallantois. Cotyledons are evident (black arrow). White flecking in the intercotyledonary placenta is calcium deposition (circle), an incidental finding (see section "Mineralization").

grows and contacts the chorion, the amniochorion is formed; on the opposite side of the calf where the amnion contacts the allantois, the allantoamnion is formed. With maturity, the allantois increases in volume to almost entirely surround the amnion.Thus the fetus is immediately surrounded by the amniotic fluid, which is produced by transamniotic fluid fluxation, fluid from the fetal lungs, oral glands, and urination. Initially the amniotic fluid is watery, slightly yellow and clear; later in development it becomes viscous, translucent, opaque, and white or yellow. Urine throughout early development is excreted through the urachus and umbilicus to the allantois, but it may also be excreted through the urethra. The contribution of urine to the amniotic fluid after 240 days' gestation in cattle has not been conclusively decided; both increasing and decreasing late-term transurethral urination has been reported [25, 37]. Amniotic fluid in full-term cattle varies from 2 to 8 l, with reported averages at 2.2 or 5–6 l. Allantoic fluid at term ranges from 4 to 15 l, with an average of 9.5–10 l [13, 46].

The umbilicus is relatively short, being about one-quarter the length of the fetus, or about 30–40 cm [13]. As such, rupture of the cord occurs during parturition when the fetal pelvis passes through the dam's pelvis [47]. The umbilicus is exclusively within the amnion and is composed of two arteries, two veins (which merge into one prior to entering the fetus), the allantoic stalk, and rarely yolk sac remnants. The umbilical arteries and veins are sheathed in smooth muscle, which contracts during parturition due to stretching, stopping blood flow after birth [37]. The umbilical arteries arise from the caudal aortae, running cranioventrally along the urachus, exiting with the allantoic stalk to supply the chorioallantois. In necropsied neonates, it is common to see swollen, purple, thrombosed umbilical arteries at the level of the urachus; this is a normal inflammatory reaction to the severing of fetomaternal circulation (Figure 56.16). Thrombosis of umbilical arteries will be absent in stillborn calves [48].

By day 45, fetal organogenesis is complete and the fetal growth stage then begins. Average weights, crown–rump lengths, and external characteristics achieved during growth are summarized in Table 56.3.

Approach to the Problem

Fetal loss in a herd may be divided into four epidemiologic presentations, which may simplify and better direct investigations. These four include (i) baseline losses, (ii) endemic losses that exceed baseline and occur consistently and

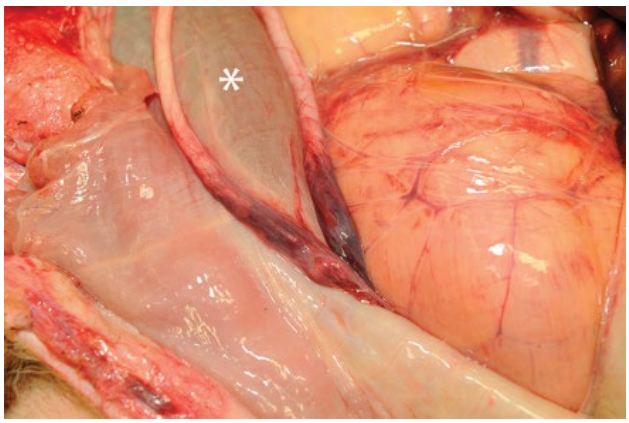


Figure 56.16 Calf with umbilical artery thrombosis. Bilaterally the arteries alongside the urinary bladder (asterisk) are focally swollen and red due to the tearing and thrombosis associated with parturition.

Table 56.3 Gestational age estimates of bovine fetuses.

Age (months)	Relative size	Crown–rump length (cm)	Weight	External characteristics
2	Mouse	6–8	8–30 g	Claw buds and scrotum present
3	Rat	13–17	200–400 g	Hair on lips, chin, and eyelids
4	Small cat	22–32	1–2 kg	Fine hair on eyebrows, claws developed
5	Large cat	30–45	3–4 kg	Hair on eyebrows and lips, testes in scrotum, teats developing
6	Small dog, beagle	40–60	5–10 kg	Hair on inside of ear and around horn pits, tip of tail, and muzzle
7	Dog	55–75	8–18 kg	Hair of metatarsal, metacarpal, and phalangeal region of extremities and beginning on back, long hair on tail tip
8	Large dog	60–85	15–25 kg	Fine short hair all over body, incisor teeth not erupted

Source: [28]. John Wiley & Sons, Inc. Original content from [13].

chronically, (iii) epidemic losses characterized by high losses in a specific time frame, and (iv) fetal losses that are confused with conception failure or neonatal losses [15].

Baseline losses, as mentioned in the Introduction, are considered largely unavoidable and may be due to illness in the dam, lethal genetic make-up of the fetus, trauma, or physiological abnormalities. It is important to monitor such losses from a management standpoint as it will aid the discovery of endemic problems. While it is prudent to thoroughly investigate all abortions, it is also costly. However, with continued monitoring and testing, the manager and veterinarian have a clearer picture of herd health and are better prepared to identify endemic problems. Furthermore, upcoming epidemics may be identified at a stage where losses can be minimized [15].

Endemic abortions significantly affect herd productivity due to chronic excessive losses. It may be difficult to realize that there is a problem, but equally difficult to identify the source of the losses. Accurate record-keeping and routine pregnancy diagnosis are therefore vitally important in order to recognize these issues [15]. Table 56.1 lists many disease-causing agents that are associated with endemic losses in cattle. BVDV, *N. caninum*, and *Leptospira* spp. are of particular importance.

Epidemic abortion storms may be due to many of the same agents that cause endemic losses. BHV-1, *B. abortus*, and *Leptospira* spp. are well-known causes [23]. Herd health status, vaccination history, pathogen virulence, access to toxic agents, and clustering of pregnancies all have relevance in determining the likelihood of epidemic loss. Herds with tight pregnancy patterns are more susceptible to this type of outbreak. Investigations should make note of the particular time/season and area where the losses are occurring as this will aid in determining differentials [15].

Finally, managers should be encouraged to document and report all abortions, stillbirths, premature births, dysmature calves, and abnormal calves.

History/Background Investigation

The importance of a thorough history cannot be overstated; essential information is found in a systematic query of farm details. Abortion is both an individual and potential herd problem, and lack of investigation in both will severely hamper efforts to alleviate unacceptably high losses. Differentiating stillbirths from abortions will help clarify if more than one problem is present [48]. It is important to keep in mind that more than one agent may be involved in abortion. Similarly, although more than one infectious agent may be present in an aborted fetus, only one may be the cause of death. Organisms regarded as low virulence may be the cause of abortion, but are also commonly present as contaminants [20].

Sporadic abortions may be a manifestation of an ongoing herd problem or they may be the beginning of an outbreak; in either case investigation is warranted. Standard forms are used to reliably collect important cow and herd information (see Appendix); systematic evaluation of management practices will help direct investigations. Pertinent questions are listed below [49]. These questions and other information are incorporated into the investigation form provided. An expanded questionnaire has been published [50].

Aborted Calf

- When was it due? Is this a stillbirth (after day 260) or abortion?

- Was it born alive?
- Are fetuses mostly male or female?

Aborting Cow

- Breed, age, lactation/parity number, source, dam/sire, bred naturally or artificial insemination (AI), conception date/last service, date of last normal parturition, previous abortions, and any work-up done.
- Vaccination history, vaccine brand/lot number, vaccine handling, and storage.
- Deworming history.
- Any clinical signs?
- Parturient period – cow movement, calving location, observation, assistance rendered?

Herd

- Number of animals, number that are immature, number purchased in last year, health problems/body condition.
- Herd abortion history (sporadic/recurrent/recent, how many in last week/month/year, which trimester affected, affects older/younger/all cows, any particular time of year), problems with term calves (congenital defects).
- Are postpartum examinations performed routinely?

Nutrition

- Feeding regime, types of forage, concentrates, minerals, selenium.
- Any changes in management, housing, pasture?
- What is the quality of the forage?
- Water source/quality?

Environment

- Access of herd to other cattle, animals, predators.
- Nature and quality of pasture.
- Any toxic plants on pasture?
- Nutrient deficiencies in area pasture?

Examination of the Cow

In some cases, the health condition of the cow has direct implications as to the cause of abortion (plane of nutrition, respiratory/intestinal disease, *Anaplasma* sp., *Babesia* sp. infection). Any vaginal discharge or cervical mucus should be collected and sent to the diagnostic laboratory for microbiological analysis. Blood samples for serology should be taken from the dam as well as unaffected cows and those that have recently aborted. Serum should be acquired two to three weeks later in order to provide paired samples. Collecting serum from ten other herdmates (or 10% of the herd) will make serological assessment more meaningful [8]. Whole blood in ethylenediaminetetraacetic acid (EDTA) is useful for blood smear and serum chemistry analysis of the dam. Gloves are to be worn during all examinations, as zoonotic agents may be present. Samples for blood culture should be taken as well.

Examination of Tissues

A necropsy is an examination with a definite purpose in order to yield the maximum amount of information. As such, it is vitally important to be systematic so that a full complement of tissues is taken during each examination. Optimally one should send the entire fetus and membranes to a diagnostic laboratory for evaluation. It is of primary importance to send fetal membranes as these may be diagnostic. If this is not convenient, then a standard approach should be adopted, as described here.

Examinations should be directed toward suspected etiologies, but keep in mind that more than one pathogen may be present. Typical findings in aborted calves that died in utero include edematous red tissues that lack normal tinctorial distinction, clear red fluids in body cavities, pale soft livers, and friable autolytic kidneys. Digital photographs are an excellent way to document lesions and may be useful in communications with diagnostic laboratories.

Examination of the Fetus

It is important to verify the approximate time of death, which can be done based on gross findings. In antepartum fetal death, tissues are autolyzed or mummified and include the findings described above. Partum death is characterized by signs of life (localized head or limb edema, partial lung aeration, limb/rib fractures, subcutaneous shoulder hemorrhage, liver fractures with hemorrhage) in addition to appropriate fetal maturation/formation. Neonatal deaths are characterized by aeration of the lungs, umbilical artery thrombosis (swollen purple/red nodules along the arteries that run lateral to the urinary bladder), loss of the eponychium (soft fetal hoof keratin), and milk in the stomachs [51].

The method of necropsy for aborted fetuses is basically the same as for any animal. The degree of autolysis and weight of the fetus and placenta are noted. Even if severely autolyzed, fetal tissues may be of use for polymerase chain reaction (PCR) screening for pathogens. Estimation of gestational age can be accomplished by the following formula: $x = 2.5(y+21)$, where x is gestation age in days and y is crown–rump length in centimeters [9]. The crown is a point midway between the orbits

on a line traversing the frontal eminence; the rump is the base of the tail or first coccygeal vertebra [52]. In embryos, the greatest total length may be used instead of crown to rump. For stillbirths, fetal weight is useful in the diagnosis of dystocia due to fetal–maternal disproportion. Also, comparison of calf weight to thyroid weight in cases of suspect hyperthyroidism (goiter) may be useful. Reports of normal thyroid weight in calves vary from 6.5 to over 18 g [53]. Further discussion on this subject can be found in the section "Non-Infectious Causes of Abortion."

Most aborted calves have no gross lesions. Small white/tan foci in the lungs, liver, and kidneys may be present and indicate necrosis or inflammation. The presence of fibrin on organ surfaces is helpful for confirming inflammation. Meconium on the perineum or in airways/stomach indicates fetal stress and anoxia, typically associated with placentitis or dystocia (Figures 56.17). Hemorrhage and fractures of shoulder and hip joints are most often seen as a result of dystocia.

Slightly raised, white skin plaques that are firmly adherent, particularly around the eyes and face, indicate fungal infection. Sometimes calves will have scattered white mineral on the hair, which is easily removed; this finding is not significant but must be distinguished from adherent fungal plaques (Figure 56.18). In abortion/stillbirth, the lungs are uninflated; they are uniformly dull red/plum colored, heavy, and sink in formalin (see Figure 56.4). In cases of dystocia where fetuses may have taken a partial breath, there may be incompletely inflated lungs; assess lung aeration in sections of cranial and caudal lobes by degree of flotation in fixative/fluid. Make note of any milk in the stomachs, as this indicates colostrum consumption, which will interfere with bacteriological and serological findings [9].

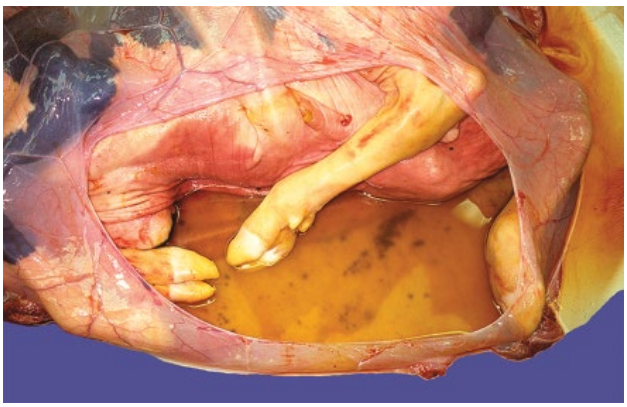


Figure 56.17 Fetus and amnion (opened). The amniotic fluid and skin are stained yellow/brown due to meconium. *Source:* Photo courtesy of J. Cooley.



Figure 56.18 Fetus with white mineral grit present over the shoulder. Not to be confused with fungal dermatitis. *Source:* Photo courtesy of J. Edwards.

Necropsy Method

It is best to have an abortion kit prepared beforehand; Table 56.4 enumerates materials to have in a kit [54]. Zoonotic pathogens may be present, so always wear gloves, protective clothing, and eye/face protection, and use careful technique when examining dams and fetal tissues. In addition, materials used in standard field necropsies are needed: clean water, bucket, brush, scale, and disinfectant. Make sure to disinfect contaminated sites and equipment. If there are questions as to which samples are best to take, contact the diagnostic laboratory to confirm which samples will be

Table 56.4 Contents for abortion kit.

Knife (15-cm boning) and steel
Nitrile/rubber gloves
String and ruler for measurements
Shears (small tree limb variety works well)
Culturette swabs
Overalls, washable apron, rubber boots
Alcohol swabs, syringes, needles to draw blood/fluids
Scalpel blades and handle
Lighter
Red-top glass vacutainer tubes, purple-top EDTA tubes
Hatchet/cleaver and hacksaw
Sterile rat-tooth forceps and scissors
Styrofoam box/cooler for temporary specimen storage
Sterile containers/whirltop bags/ziplock bags, permanent pen
Fixative (10% neutral buffered formalin), at least 500 ml in plastic sealable container/jar
Bucket, brush, disinfectant

submitted for advice. Taking photographs of lesions is a relatively simple and excellent practice which will aid diagnosis.

1) Measure crown–rump length along the vertebral ridge (see section "Examination of the Fetus") and weigh fetus and placenta separately. Examine skin for exudate and abnormalities, around the eyes and face in particular for signs of fungal infection. If skin lesions are present, perform scrapes for fungal culture/wet mount examination.
2) Note hair development/whiskers and muscle tone.
3) Examine the body for congenital abnormalities, umbilical hernias, cleft palates, facial anomalies, etc.
4) Examine for hemorrhages, fractures, hypopyon/hyphema, and lingual edema.
5) Wash the surface of the fetus with water to remove gross contaminants.
6) With the left side down, reflect right legs with a knife and continue the skin incision from cranial to caudal, reflecting the skin to the dorsal and ventral midlines. Collect any pooled blood between incised tissue planes with a syringe if available (for serology).
7) Open abdomen at the highest point along the caudal aspect of the ribs, being careful not to contaminate any peritoneal fluid or tissues. Aspirate any abdominal fluids with a sterile syringe, place in sterile tube, label, and refrigerate. Such fluids may be used as fetal sera. Continue the cut along the caudal aspect of the ribs, along the lumbar area, and cranial to the pelvis to expose the abdominal viscera. Note any lesions, and volume, character, and color of fluids.
8) Incise the diaphragm (noting if negative pressure is present) and cut the ribs at the costal angles and along the sternum; remove the chest wall. Aspirate any thoracic fluids with a sterile syringe, place in sterile tube, label, and refrigerate. Such fluids may be used as fetal sera. Note any lesions, and volume, character, and color of fluids. Pleural surface of the removed chest wall may be used as a makeshift cutting board/sterile field for dissection.
9) Aspirate fluids from the unopened abomasum with sterile syringe, place in sterile tube, label, and refrigerate. Note the character of the abomasal fluid. Take samples of any other suspicious fluids at this time.
10) Examine the body cavities and organs in situ, making note of fibrin, hemorrhages, or abnormally situated organs.
11) Remove thoracic viscera from tongue to lung (pluck). Examine the mouth; one may split the mandibular symphysis with a knife. Incise the esophagus along the entire length; incise the dorsal tracheal membrane from the larynx to the bronchi. Examine the chest for any fractures. Slice multiple lung lobes, examining the cut sections for changes in the lobules and airways. Examine the thyroid gland. Collect tissues, especially lesions (see numbers 18 and 19).
12) Examine the heart, making sure to inspect all four main valves and four chambers. Look for ventricular septal defects or other anomalies. Heart blood may be used for serology testing.
13) Collect visceral organs, examining gastrointestinal tract last. Identify the kidneys, ureters, and urinary bladder. Remove the gastrointestinal tract by incising the root of the mesentery and continuing the cut to the diaphragm along the dorsum, avoiding the urinary tract. The tract may then be lifted out of the abdomen, leaving the colon attached and uncut. Collect tissues, especially lesions (see numbers 16–18).
14) Remove the adrenal glands.
15) Remove the urinary tract, making multiple slices through the kidneys. Identify genital organs.
16) Serially section the liver and spleen.
17) Open the forestomachs and abomasum and examine for content. Continue into the intestine, making several incisions along each major section (duodenum, jejunum, ileum, cecum, colon). Examine content and the mucosa.
18) Collect organs (kidney, liver, lung, spleen, thymus, adrenal) in sterile bags (placenta kept in separate bag), and refrigerate.
19) *Collect in formalin for histopathology: lung, liver, kidney, spleen, heart, adrenal glands, skeletal muscle (three to four sections including tongue, diaphragm), thyroid, ileum, skin (including eyelid), thymus, and mesenteric lymph node in 5-mm sections.*
20) Remove the head from the body at the atlanto-occipital joint. Harvest the entire brain (can be done at the clinic), even if severely autolyzed, and place whole in formalin. If the fetus is severely autolyzed, take a swab culture of the brain tissue prior to fixation. The skull is relatively soft and can be easily opened with a saw, making two cuts along the inner aspects of the occipital condyles, cranially (and somewhat laterally along the curve of the calvaria) toward the inner aspects of the orbital rims. Make a third incision across the orbital ridge, connecting the first two cuts. Pry the cranium from the skull in one piece. Flip the head over, placing the dorsum of the brain in the palm of your hand and, with a scalpel, gently cut the nervous attachments of the base of the brain, allowing the brain to fall into your hand.
21) Collect ocular fluid with a needle/syringe for nitrate/nitrite levels if needed, and freeze. Collect eyelid and ear notch for histopathology.

22) Open several joints, looking for purulent exudate, fibrin, and hemorrhage.
23) Examine fetal membranes for exudates, lesions, and abnormalities (see next section for detailed instruction). Remove dirt/debris from the membranes prior to sampling.
24) Place two to three sections with cotyledons in a sterile bag, and refrigerate; make impression smears of any lesions.
25) Harvest the placenta for histopathology (at least three to four sections including cotyledons and intercotyledonary areas).
26) Collect amniotic fluid if available, refrigerate, and culture.
27) Collect dam blood in red-top tube for serology (herdmate blood samples may be collected as well; ten cows or 10% of the herd has been suggested). Blood in EDTA can be used for PCR.

Examination of the Membranes

Collect as much of the membranes as possible and carefully examine the chorioallantois and amnion, as lesions may be subtle, focal, and easily overlooked. In many cases, little if any placenta can be examined. It is important to try to verify if all membranes have been expelled. Because of the attachments of the amnion to the chorion, as well as the short umbilicus, calves will rupture and escape both the amnion and chorioallantois at delivery. Typically, membranes are found with cotyledons on the outside.

The fetal membranes may be laid out lengthwise to identify the umbilicus which is continuous with the internal aspect of the amnion. Examination of amniotic membranes and fluids is best done at this time. The umbilicus is composed of four muscular turgid vessels (arteries and veins) loosely enmeshed in slippery membranes. The gravid horn is easily identified as the larger half of the tissue and should have larger placentomes. Avascular chorionic tips (necrotic tips) should be identified and inspected carefully, as true placental lesions may be present in the gravid horn near the tip [19]. The umbilicus contains the umbilical arteries and veins, which form a useful longitudinal axis by which to orient the placenta. From there the internal aspect of the allantoic cavity may be examined, with attention to the nature of any fluids present, thickness and color of membranes, and peculiar odors. Allantoic calculi are typically found at this time.

By placing the umbilical vessels at one side of the tissue, a lengthwise incision along the opposite side of the placenta will allow the placenta to be opened and laid flat, with the umbilical vessels forming the central axis (Figure 56.19). The horns are unequal in size, the gravid horn being much larger. In this way, vessels and cotyledon rows (normally four) may be examined thoroughly. Fresh cotyledons have a red velvety texture with a mildly irregular surface. The size variation, shape, color, consistency, and degree of cotyledon mottling should be noted, as well as degree of autolysis. Occasional small tags of attached caruncular tissue may be seen. Cotyledon margins are typically sharply defined, and the intercotyledonary chorion is white, translucent, and smooth. Adventitial placentation is a frequent finding that appears as small cotyledons or villous tuft formations adjacent to the placentomes that may cover the entire placenta. Abnormalities in the placenta are described in the following sections.

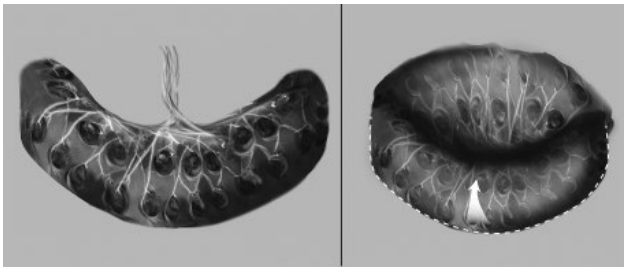


Figure 56.19 Placental membrane examination method. (*Left*) The long axis of the chorioallantois is arranged perpendicular to the umbilical stalk. (*Right*) An incision is made along the dotted white line, allowing the chorioallantoic sac to be opened (direction of white arrow) and laid flat. In this way, the vascular axis, cotyledons, and membranes can be thoroughly examined. *Source:* Courtesy of Rachael Fishman.

Sample Collection

A complete set of tissues should be collected in every case [55]. Depending on the etiologic agent (particularly bacterial), different organs may have significantly different recovery rates; lung, liver, and abomasal fluid should all be submitted for culture/examination to maximize success [56]. Biological specimens must be properly collected, prepared, stored, and transported, otherwise diagnostic accuracy will suffer. It is important to maintain aseptic technique despite apparent tissue contamination; this is necessary to maximize chances for diagnosis as well as for prevention of zoonotic infection. Microbiology samples should be kept chilled and sent to the diagnostic laboratory as soon as possible in a Styrofoam cooler with ice packs for overnight courier shipping. If fresh tissue samples cannot be sent within two to three days, freezing at −70 °C and delivering on dry ice is ideal [55]. If necessary, specimens can be placed at −20 °C until dry ice can be obtained. Conventionally frozen tissues (−20 °C) are often acceptable for PCR testing of many pathogens. Abomasal and thoracic/pericardial fluid samples should be sent in sterile

tubes without additives. Frozen fetal liver, lung, and kidney (2.5–5 cm chunks, −20 °C) should be kept for testing in case nutritional or toxicologic disease is suspected after initial examinations [49].

Chilled or frozen tissues should be placed in sealed bags (whirlpack-type bags often leak), preferably with a secondary bag around them. Fluids shipped in glass tubes require ample padding in tight-fitting containers. Absorbent materials should also be included in the package to prevent soaking of the exterior, which complicates and may delay the shipping process. Disposable diapers are an effective option [49]. Contact your diagnostic laboratory for proper packaging and shipping requirements for abortion samples, as they may contain infectious and/or zoonotic agents.

Viruses may have very specific tissue tropisms and therefore require certain tissues for isolation. Specimens need to be collected as soon as possible as autolysis can be very damaging to virions. Virology samples for abortions are typically pooled, although placing tissues in separate bags is ideal. Placental tissues should be bagged separately from fetal tissues. Do not pool samples from multiple abortions. Tissues should be kept cool but not frozen until arrival at the diagnostic laboratory. If tissues will not be immediately used, freezing at −70 °C is better than at −20 °C [49]. If live viruses are sent packed in dry ice for virus isolation, be sure to place samples in airtight containers as the gaseous phase of dry ice is carbon dioxide, which can lower sample pH and inactivate some viruses. Certain laboratories may have preferences for virological samples, particularly if a certain pathogen is highly suspected.

Bacteriology tissue samples should be 2.5–5 cm per side in order that surfaces may be heat seared for proper culture. External and gut samples should not be bagged with internal organs. If samples are contaminated, sending a swab culture (Culturette™) in addition to tissues may be helpful. For severely decomposed specimens, aseptically collected brain tissue may be useful for isolating pertinent organisms [26].

Fungal sampling should at least include affected cotyledons; however, it is best to send the entire placenta. Fetal infection is inconsistent, but lung, skin, and abomasal contents are useful.

For histopathology, sections approximately 5 mm wide in 10% neutral buffered formalin are best. The brain, however, should be immersed whole in formalin, without dissection. If the brain is liquefied, fix separately. For small lesions, several foci should be submitted. For large lesions, a few sections including the margin with normal and abnormal tissues should be collected. Identify clearly any lesions seen in order to alert the diagnostician.

Tissue smears from abomasal content or placenta may assist diagnosis; wet mount examination and preservation of air-dried smears can prove valuable for detecting bacteria, fungi, and protozoa.

At least 10 volumes of formalin per volume of tissue are needed for proper fixation. Tissues should be allowed to fix for at least 24 hours, preferably 3 days. The brain in particular requires ample fixative over 3–4 days. Addition of 1 part ethanol to 9 parts formalin (10% ethanol, 90% formalin) may be used to prevent freezing in cold climates [57]. If formalin is not available, 70% ethanol may be used. Tissues may be sent in formalin, otherwise fix tissues for 24 hours, drain, and send in bags with formalin-moistened gauze. Be sure to double-bag samples as containers may rupture in transit. Contact your diagnostic laboratory for instructions.

For blood samples, blood from the dam as well as the fetus is ideal. Fresh blood smears are particularly useful if anaplasmosis is suspected. Blood in EDTA is preferred for PCR testing. For serum samples, draw 8–10 ml of serum into tubes without additives, allow to sit for one to two hours until the clot begins to retract, then place in the refrigerator overnight. Centrifuge at 1000×*g* for 10 minutes and decant serum; send in a separate sterile tube. One may freeze the serum (do not freeze whole blood) until needed.

Table 56.5 lists typical sample storage conditions.

Table 56.5 Fresh specimens collected for fetal and neonatal diagnostics.

Test	Storage	Specimens	Notes
Bacteriology/mycology (culture, PCR)	Keep refrigerated *not* frozen unless PCR only	Stomach contents, pericardial fluid, liver, lung, brain, placenta	Collect stomach contents or pericardial fluid in a syringe with a large-gauge needle Package each specimen in separate containers. Placental culture results are often contaminated – evaluate critically
Virology (virus isolation, fluorescent antibody test, PCR)	May be frozen (at −70 °C if necessary)	Lung, liver, kidney, heart, blood, placenta	Package each specimen in separate containers
Nutrition, toxicology	May be frozen	Liver, kidney, ocular fluid	
Fetal/maternal fluids	Keep refrigerated	Dam serum/fetal fluids	Paired (acute and convalescent) if possible

Diagnosis

Arrival at an accurate diagnosis is the result of the cooperative efforts of the attending veterinarian and the diagnosticians at the laboratory. Even with the submission of proper samples, the establishment of a definitive diagnosis is problematic. Expulsion of a dead fetus often occurs hours to days after death; the resulting autolysis makes the difficult task of fetal lesion identification even more so. Fetal membranes, which may have the only significant lesions to be found, are often not present for examination. Also, in many cases examined tissues are severely contaminated, whether due to incomplete expulsion or from the environment. Finally, diagnostic laboratories run routine tests to identify well-known causes of abortion in a timely manner. As such, investigation of unusual or poorly understood etiologies is beyond their purview [20].

Keep in mind that the majority of pregnancy wastage occurs at earlier stages of development, when expelled tissues are least likely to be noticed. Early gestation abortions are often detected only weeks after the fact, making investigation and diagnosis all but impossible. The majority of tissues examined by clinicians and diagnostic laboratories are of large fetuses, which may bias the interpretation of a herd problem where losses occur at various gestation stages. Late-stage abortions may be the result of injury many weeks prior to expulsion; they may not exhibit important or telling changes that could be present in earlier aborted fetuses, preventing accurate diagnosis. Worse yet, in an effort to find a cause, other coincident agents may be erroneously blamed, depending on what is found in the submitted fetus [8]. A positive result for a pathogen using only one test modality may lead to error; serology, tissue smears, PCR, and histopathology should all be utilized in order to reach a confirmatory diagnosis [58]. PCR is an optimal first-line approach.

It is important to realize that more than one disease or management problem may be at work in a herd, complicating the resolution of endemic problems. Multiple pathogens may be present; some may not be obvious, while those that are readily evident may not be directly contributing to abortion. In these cases, determining the actual cause of abortion is challenging.

Serology results must be cautiously interpreted. A positive sample from a cow indicates exposure, which may or may not be relevant in light of vaccination status. Twofold to fourfold increases in titer over two weeks may be significant, particularly with herdmate samples for comparison [8]. However, it is likely that dam serum antibody levels have reached their peak by the time abortion occurs, so it is not unexpected to see a lack of rise in titer two to three weeks later [15]. Serology may be particularly useful in diagnosing BVDV, leptospirosis, anaplasmosis, and abortions where the agent is difficult to demonstrate by other methods [20]. The presence of a specific antibody in fetal serum does not mean that particular agent is the cause of abortion; BVDV and *N. caninum* are two examples. Serology results must be carefully weighed with microbiology, histopathology, molecular testing, and herd history data. Table 56.6 lists common diagnostic tests for specific pathogens.

Table 56.6 Summary of diagnostic tests for bovine abortion.

Agent	Preferred fetal tissues	Preferred fetal diagnostic test	Additional diagnostics
Bovine herpesvirus 1	Kidney, adrenal, liver, lung	FA (frozen tissue), IHC, VI	IHC of placenta if placentitis is present
Bovine viral diarrhea virus	Lung, heart, kidney, placenta, skin	FA, IHC, VI, PCR	Antigen-capture ELISA, RT-PCR
Bluetongue virus	Brain, spleen	PCR, VI	Fetal serology
Bunyaviruses (including Schmallenberg)	Brain	PCR	Fetal/precolostral serology; congenital abnormalities
Brucella	Placenta, lung, abomasal contents, uterine fluid	Bacterial culture	Culture of milk from dam; dam and fetal serology
Listeria	Placenta, lung, brain, abomasal contents	Bacterial culture, IHC	Gram's stain of freshly aborted tissues, PCR
Salmonella	Placenta, liver, lung, abomasal contents	Bacterial culture	

(*Continued*)

Table 56.6 (Continued)

Agent	Preferred fetal tissues	Preferred fetal diagnostic test	Additional diagnostics
Yersinia pseudotuberculosis	Placenta, liver, lung, abomasal contents, intestines	Bacterial culture	
Leptospira	Kidney, placenta	FA (fetal kidney smear), IHC, PCR	PCR of fetal urine; fetal and maternal serology
Ureaplasma	Lung, abomasal contents, placenta	*Ureaplasma* culture, PCR	
Campylobacter	Lung, abomasal contents, placenta	*Campylobacter* culture, IHC, darkfield microscopy	Silver staining of histology tissues, PCR
Epizootic bovine abortion	Thymus, spleen, lymph nodes	Modified Steiner's silver stain of histology slides, IHC	Elevated fetal serum immunoglobulins
Chlamydia	Placenta	PCR, IHC, FA	Macchiavello's, Gimenez, or modified acid-fast stains of histology slides
Coxiella burnetii	Placenta	PCR, IHC	Macchiavello's, Gimenez, or modified acid-fast stains of histology slides
Fungi	Placenta, abomasal contents, lung, skin lesions	Fungal culture, H&E	Direct identification by KOH wet mounts of skin or placenta or GMS and PAS stained histology slides
Tritrichomonas	Placenta, abomasal contents, lung	*Tritrichomonas* culture, Bodian's silver stain of histology slides, IHC, H&E	Darkfield microscopy of abomasal contents, PCR. Examination of maternal uterine discharge
Neospora	Brain, lung, kidney, skeletal muscle, liver, placenta	IHC, PCR	Fetal serology (IFA, microagglutination titer, ELISA). Maternal serology
Sarcocystis	Brain, lung, liver, kidney, skeletal muscle, placenta	IHC	Genomic probe; ribosomal RNA assays
Nutrient deficiency	Liver, heart, thyroid	Thyroid iodine assay Liver mineral (selenium)	Maternal blood-selenium, other
Congenital defects	Fetal hair	Fetal muscle	Teratogenic virus testing
Toxicosis	Liver, kidney, fat, urine, brain Abomasal/amniotic fluid, thoracic fluid	Gas chromatography mass spectrometry	

Source: Based on [28].
ELISA, Enzyme-linked immunosorbent assay; FA, fluorescent antibody test; GMS, Gomori–methenamine silver stain; H&E, standard histopathology stains; IFA, indirect fluorescence test; IHC, immunohistochemistry (usually formalin-fixed tissues); KOH, potassium hydroxide test; PAS, periodic acid–Schiff stain; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; VI, virus isolation.

Specific Manifestations of the Conceptus Relating to Pathology

Mummification

Mummification is an uncommon event in which there is often no certain cause. It is the process of progressive dehydration and compaction of a sufficiently mature dead fetus in utero. In order for this to occur, bacterial infection (tissue lytic organisms) of the dead fetus must be absent, no air can be present in the uterus (closed cervix), and a functional CL must be present. The time course, which may take months, depends on the size of the fetus. This process generally occurs in the second and third trimesters, when fetal bones are sufficiently developed to resist resorption. As fetal fluids are absorbed, tissues become compressed, shrunken, red/brown, and sticky to dry, without odor. As the membranes atrophy and the uterus involutes, hemorrhage occurs between the uterus and fetal tissues, imparting the red "hematic" sticky material onto the tissues (Figures 56.20 and 56.21). This hematic type of mummification apparently is distinctly bovine [13, 36].



Figure 56.20 Opened uterus with mummified fetuses. Friable, non-odorous, hematic material obscures the fetuses. *Source:* Photo courtesy of J. Edwards.



Figure 56.21 Fetal mummy. The carcass is shrunken and contracted with diffuse red/brown discoloration, dry tissues, and sunken eyes. Tissues lack odor and emphysema. *Source:* Photo courtesy of J. Edwards.

The uterus tightly wraps around the fetal materials, compacting the mummy. The longer this proceeds, the drier and firmer the fetus becomes, remaining in utero for as long as two years. In the cow, mummification is most common at the end of the first and beginning of the second trimesters [60]. Various infectious and non-infectious etiologies have been implicated in their occurrence (BVDV, tritrichomoniasis), but the cause is usually unknown [12, 19]. Mummification may be associated with certain breeds (Guernsey, Jersey) or certain breeding/genetic combinations [13]. In cases of uncomplicated mummification, prognosis is good for return to fertility [36].

Maceration

Maceration describes the effect of dead fetal tissue softening due to fluid soaking and saprophytic digestion in utero

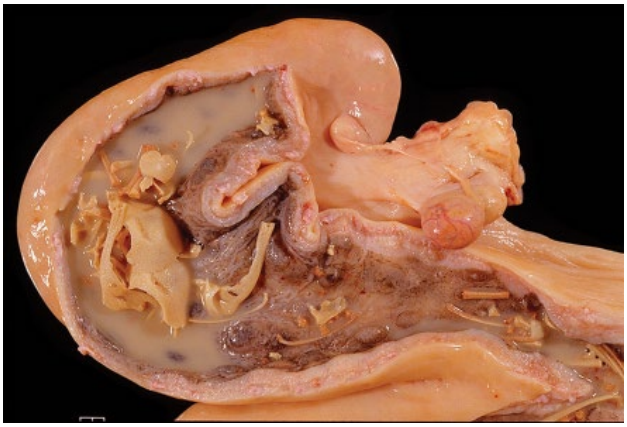


Figure 56.22 Opened uterine horn with a macerated fetus. Bone fragments in purulent fluid remain. The ovary has a retained corpus luteum. *Source:* Photo courtesy of J. Cooley.

(Figure 56.22). In cattle this is due to exposure of the retained fetal tissues to bacteria. This occurs when there is failure of fetal expulsion after death with retention of the carcass partially or entirely within the uterus. The bacteria may be those that caused fetal death or those that arrive through an open cervix [12]. Maceration is distinct from autolysis, which is digestion of tissues by endogenous enzymes and requires no bacteria. When bacteria gain access to a dead fetus in the reproductive tract, they are able to multiply rapidly at body temperature and are relatively insulated from the dam's immune system. Bacterial digestion of tissues and gas formation (emphysema) ensues. The uterus surrounds the fetus and there is an intense metritis.

In early gestation, macerated embryos are resorbed or expelled with little exudate. After the first trimester, the fetal skeleton is sufficiently formed to resist disintegration, and skeletal fragments may be retained. Bone fragments may become embedded in the uterus or may perforate the wall and remain free in the abdomen [60]. CL regression and cervical dilation may be found on palpation. Cows may display vague signs of illness due to a persistent and severe uterine infection [19].

Emphysema usually occurs when incomplete abortion or near-term dystocia allow putrefactive vaginal flora to invade the dead fetus. Incomplete abortion occurs when there is partial cervical dilation and insufficient expulsion of the fetus, which is partly or totally retained in the uterus [19].

Embryonic Death with Persistent Membranes (Moles)

Moles are not neoplasms, but are masses of hyperplastic or edematous immature placental tissue [61]. Once fertilization occurs, the blastocyst divides into embryonic and extraembryonic tissues; the latter becomes the trophoblast, which is

the forerunner of the placenta. Later in development, endodermal and mesodermal tissues from the embryo migrate along the inner aspect of the trophoblast to form the chorion, which is the outermost fetal tissue that is in contact with the uterus. Thus, embryonic mesenchyme contributes to the stroma of the placenta. This is an important point, for in order to form placental tissues, embryonic tissue must have been present [61, 62]. Thus it is not surprising to find fetal tissues in moles, even though it is rare.

Hydatidiform moles are defined in humans by macroscopic features and are termed (complete and partial) based on the degree of fluid accumulation. They are named hydatidiform for their abundant, bulbous, botryoid to polypoid villi, which are edematous with watery fluid-filled cisterns. Villous edema and trophoblastic hyperplasia are constant and characteristic features [61, 63]. The hydropic swelling seen in moles may also occur in unrelated gestational disorders, such as maldevelopment of the placental vasculature or edema following early fetal death [64].

In humans, complete moles are derived from an anucleate egg fertilized by one or two sperm, leading to a paternally derived (androgenetic) tissue, most often 46XX. Conversely, partial moles arise from the fertilization of an ovum by two sperm, generating a triploid conceptus [61]. Such anomalies lead to fetal loss, but membranes survive and proliferate (mole).

Moles in cattle are rare and have been reported as hydatidiform or cystic [19, 65–67]. Bovine placental masses similar to hydatidiform moles in humans have been reported to be between 40 and 60 cm wide and weighing up to 48 kg [66]. The masses are smooth, soft/firm, ovoid/irregular, and pale gray/tan with multiple pendulous nodules that appear fatty. They are often associated with twinning [66, 67]. Microscopic descriptions resemble descriptions in the human literature [67, 68]. Recently, a mole of androgenetic origin (60XX) was reported in association with a stillborn male calf [66].

More commonly, moles in cattle have been termed "cystic placental moles." They are thought to arise after fetal death, with or without resorption, where membranes persist as an empty cystic structure of a size similar to normal membranes at three to four months' gestation [19, 65]. The cyst is filled with clear gel-fluid and stroma. In some cases, the tissues become infected, leading to pyometra and expulsion [19].

Acardiac Twins

Acardiac twins (acardiac monster, amorphous globosus, holoacardius acephalus, holoacardius amorphous) are the most bizarre and severely malformed fetuses imaginable. They represent a twin whose development is completely disturbed and, unlike the name, may indeed have some cardiac tissue remnants. Despite this, a functional heart is absent. They develop in association with a twin that has sufficiently normal cardiovascular function to support the monster through placental vascular anastomoses [69]. Various theories as to their development have been proposed [70]. In cattle, such twins often manifest as globoid, hair-covered masses of soft tissue attached to an umbilicus (Figure 56.23). Other monsters have rudimentary limbs, faces, and teeth. In rare cases multiple monstrous anomalies may be present [13].

Adventitial Placentation

In some cases, particularly in older multiparous cows and those with a history of metritis, there is inadequate placentomal development due to uterine damage. To compensate, the intercotyledonary chorion develops bright-red to tan, fine, villous structures and small accessory cotyledons (adventitial placentation) (Figures 56.24 and 56.25). In some cases proliferation may be abundant [13]. Rarely, villous proliferation may appear as a diffuse process with few or no obvious placentomes due to destruction of caruncles [71, 72]. This process may be incidental, but excessive adventitial placentation may be associated with pathologic conditions such as hydrallantois [19, 73]. This condition indicates subnormal placental capacity and such cows, particularly if other stressors are involved, may not be able to maintain full-term calves, resulting in late-term abortions/stillbirth.

Dropsy of the Fetal Sacs (Hydramnios/ Hydrallantois)

Hydramnios and hydrallantois refer to excessive accumulation of fluids in the amnion and allantois cavities, respectively;



Figure 56.23 Acardiac monster with umbilicus. A hair-covered soft tissue globe. *Source:* Photo courtesy of J. Cooley.



Figure 56.24 Chorioallantois, maternal aspect, with adventitial placentation. Placenta (top) has been partially pulled away from the endometrium (bottom). Cotyledon proliferation is irregular but tissues are shiny, without fibrin, intercotyledonary hemorrhage, or necrosis. *Source:* Photo courtesy of J. Cooley.



Figure 56.25 Chorioallantois, fetal aspect, with adventitial placentation. Arteries and veins (white) arborize onto the coalescing placentomes. *Source:* Photo courtesy of J. Cooley.

edema of the chorioallantoic membrane may also be present. In most cases, aside from Dexter bulldog calves, fluid accumulation occurs in the last trimester [36]. Fetal swallowing partly regulates amniotic fluid volume in the second half of gestation; conditions that prevent or slow fetal swallowing may lead to fluid retention [45]. Allantoic fluid is a watery fluid generated by the fetal kidney. It is excreted through the urachus, reaching 8–20l at term. Amniotic fluid is more viscous and glairy (albuminous), due in part to fetal salivary secretions.

Hydrallantois is associated with placental dysfunction and is seen with uterine or placental disease. It is generally considered a maternal placentation problem and adventitial placentation is commonly present [74]. This condition is seen sporadically in dairy and beef cattle and has been reported with *Sida carpinifolia* toxicosis in Brazil [75]. It is more common in twin pregnancies. Membranes may be more fibrous than normal, but the character of the fluid is grossly normal. Fluid accumulation may reach 250l. [74] Fetuses are usually born dead or are small and may have ascites or anasarca (Figure 56.26) [19, 73]. A heritable condition was identified in Japanese Black cattle in which fetal polyuria led to hydrallantois and secondary pressure-induced hydronephrosis, a correlation which has been reported in other breeds [76, 77].

Hydramnios is much less common than hydrallantois. It is associated with fetal malformations that may be congenital or inherited. Congenital defects include any severe anomaly, such as schistosomus reflexus, that impairs normal swallowing. Inherited defects include Dexter bulldog fetuses (see Figure 56.12), hydrocephalic Hereford fetuses, Angus fetuses with osteopetrosis, and Guernsey fetuses with pituitary hypoplasia [13].

Fetal Dropsy

Fetal dropsy encompasses three main presentations: anasarca, ascites, and hydrocephalus. Fetal anasarca may be seen with dropsy of fetal sacs (Figures 56.26 and 56.27). It is sometimes associated with genetic defects such as pulmonary hypoplasia. Fluid accumulates in soft tissues, often enlarging the fetus such that cesarean section is required [78]. Fetal ascites may be commonly seen in cases of infectious abortion or developmental defects. Ascites or placental edema may occur as a rare accompaniment to *B. abortus* infection [13, 36].

Hydrocephalus

Hydrocephalus refers to an absolute increase in cerebrospinal fluid (CSF) within the brain ventricular system, within the meninges, or both (Figure 56.28). Congenital hydrocephalus



Figure 56.26 Fetus and placenta. The fetus is edematous (anasarca) with yellow/brown skin due to meconium staining. The allantois is markedly expanded by edema (hydrallantois). *Source:* Photo courtesy of J. Cooley.



Figure 56.27 Fetus with anasarca. *Source:* Photo courtesy of J. Edwards.

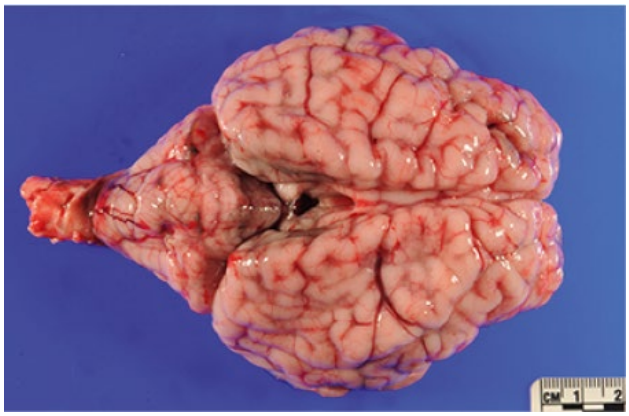


Figure 56.28 Brain, dorsolateral aspect. Hydrocephalus, with collapse of cerebral hemispheres and cerebellar coning.

occurs in two main forms: obstructive (hypertensive) and compensatory (normotensive). Obstructive hydrocephalus is associated with an inherited malformation, the most common of which is stenosis of the mesencephalic aqueduct [79]. Six such heritable forms have been described in Holstein, Jersey, Hereford, Charolais, Ayrshire, Dexter, and Whitebred Shorthorn breeds. Affected calves often have many other obvious defects [80]. Characteristically, there is marked doming of the skull with malformation of the bones of the cranium [81].

Compensatory hydrocephalus, on the other hand, arises as a result of CSF accumulation after severe brain necrosis. It is often associated with in utero infections with BVDV and Akabane, RVF, Wesselsbron, Schmallenberg, and bluetongue viruses. Hydranencephaly is a form of compensatory hydrocephalus where the neopallium (outer cerebrum) has been reduced to a very thin, almost translucent membrane with little to no parenchyma (see section "Bovine Viral Diarrhea Virus (BVDV) Infection"). Such lesions result from the aplastic and necrotizing effects of viral infection. The brainstem, cerebellum, hippocampus, and olfactory regions may be spared. The bones of the skull cavity are generally normal [79]. Other causes of congenital hydrocephalus include *Veratrum californicum* ingestion by pregnant cows and vitamin A deficiency [82]. The cause of hydrocephalus may be difficult or impossible to determine in some cases.

Amniotic Plaques

Amniotic plaques are normal, discrete, flat, white, 2–20 mm wide, round to irregular foci of squamous epithelium on the internal surface of the amnion (Figures 56.29 and 56.30). Immediately around the umbilicus they may become papillary or villous. They often resolve by six months' gestation, occur most often around the umbilicus, and are not found on the fetus. Plaques are not considered to be of pathologic significance and are not associated with



Figure 56.29 Fetus and placenta (amniotic surface exposed). The umbilicus and amnion are covered by amniotic plaques. *Source:* Photo courtesy of J. Cooley.



Figure 56.30 Placenta, umbilicus, and fetal aspect of amnion. Numerous coalescing amniotic plaques cover the amnion. *Source:* Photo courtesy of J. Cooley.

signs of inflammation. They are to be distinguished from fungal plaques. Their presence is useful for orientation of tissues for examination as they occur on the internal (fetal) side of the amnion [19].

Mineralization

Mineralization of the allantois or amnion appears as a network of white spots or streaks, typically associated with small vessels (see Figure 56.15). The material is not gritty but is soft, occurring most often in the second trimester [19]. After this time the mineral disappears and is probably incorporated into fetal bone [13]. Mineral may also be present on the coat of the calf which may be easily wiped from the hair (see Figure 56.18). The origin and significance of this finding is uncertain, but it must be differentiated from fungal dermatitis.

Necrotic Tips

Necrotic tips (Figure 56.31), or avascular chorion, is a normal area of necrosis occurring at the extreme tips of the chorioallantois of both horns. These areas are pale tan, shrunken, up to a few centimeters long, and clearly demarcated from the villous chorioallantois. Necrotic tips may be unilateral, particularly in the non-gravid horn. In some cases, necrosis of the non-gravid horn may involve as much as the entire horn [45].

Allantoic Calculi (Hippomanes in Horses)

Known since the time of Aristotle (*hippomane* means "horse madness"), these allantoic aggregates are composed of mineral and mucoprotein that deposit in progressive laminations around a nidus of cell debris (Figure 56.32) [83].



Figure 56.31 Chorioallantois with uterine horn tips. The horn at the top is avascular and shrunken. The lower horn tip is relatively normal. *Source:* Photo courtesy of J. Edwards.



Figure 56.32 Hippomane and avascular chorion tip. *Source:* Photo courtesy of J. Edwards.

Allantoic calculi are irregular, discoid, rubbery, tan masses with tapering margins, 3–15 cm wide and up to 4 cm thick [13]. They have been described in horses, donkeys, zebras, cattle, deer, goats, and ewes, among others [84]. In cattle they are common and may be multiple. They are not known to be of significance.

Meconium

Meconium is a combination of sloughed intestinal and epidermal cells, mucus, bile acids, and various other metabolites of fetal development. These contributed materials collect in the fetal colon, where water and electrolyte absorption forms thick, brown, tenacious feces that may develop into pellets. Calves will also shed hair into the amniotic fluid, which is then swallowed by the fetus and collected in the colon to be expelled as part of the meconium. As such, meconium pellets may be composed of abundant matted hair, which may be expelled into the amniotic fluid or may be ingested and found in the alimentary tract of the fetus [39, 85]. In cases of fetal stress/hypoxia, meconium may be prematurely expelled and aspirated, causing discolored amniotic fluid, smearing of meconium on the fetus, and meconium in airways (Figures 56.26 and 56.33).

Arthrogryposis

Arthrogryposis describes a condition where congenital joint contractures occur in one or many limbs and may be associated with scoliosis, kyphosis, lordosis, and torticollis. The limbs are rigidly fixed and variably bent in every sort of unnatural position. These changes occur as a result of decreased limb movement due to myogenic or neurologic abnormalities. As a result of neurologic disease in utero, muscles are underdeveloped and articular surfaces are

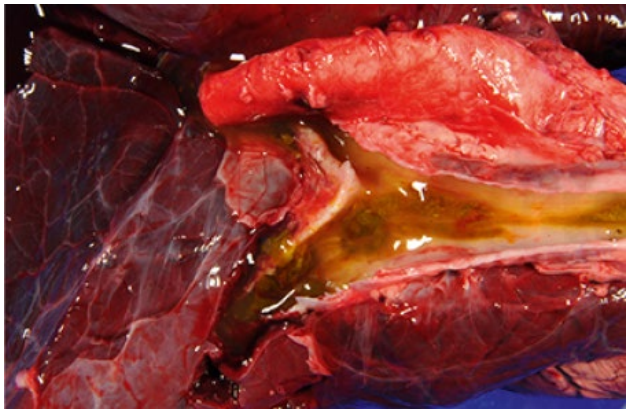


Figure 56.33 Meconium aspiration; stillbirth due to severe neurologic malformation (Chiari-like).

misshapen due to lack of proper stimuli. It may occur sporadically, in association with viral infection (bluetongue, Akabane, BVDV, Schmallenberg), or due to maternal toxin ingestion (lupines, poison hemlock, some types of tobacco). Breed-associated syndromes in cattle have been described. Arthrogrypotic fetuses are associated with stillbirth, hydramnios, and dystocia for obvious reasons [86].

Congenital Skin/External Lesions

A variety of abnormal conditions may present in the skin and can be hereditary, but not necessarily so. Hypotrichosis/alopecia, a less than normal amount of hair, is one of the best described and many heritable forms are known [87]. Non-genetic causes include iodine deficiency (fetal goiter), pituitary hypoplasia, maternal ingestion of *Veratrum album*, BVDV infection, and autoimmunity [88]. Calves may lack hair entirely or have thin, soft, and even curly hair. Affected areas are usually symmetric and the skin itself appears normal. The distribution can be patchy and calves may also have dental anomalies. A rare and distinctive condition is ichthyosis fetalis, in which generalized alopecia is combined with marked hyperkeratosis, resulting in fissures or plate-like folding of the skin over the entire body. Calves may be stillborn or die soon after birth; it is hereditary [87].

Defects, tears, and ulcers in the skin at birth can be due to various conditions. One of the more common is spina bifida (spinal dysraphism), a congenital defect in spinal development. In its most mild form calves have small areas of alopecic or glabrous skin on the dorsal lumbar midline (Figure 56.34). The underlying muscle, bone, and spinal cord have varying degrees of malformation, with a lack of the fusion normally seen in neuraxial folding. Lesions may bulge due to meningomyelocele formation. In some cases the spinal canal is continuous with the skin and in severe cases there are anomalies of the head as well.

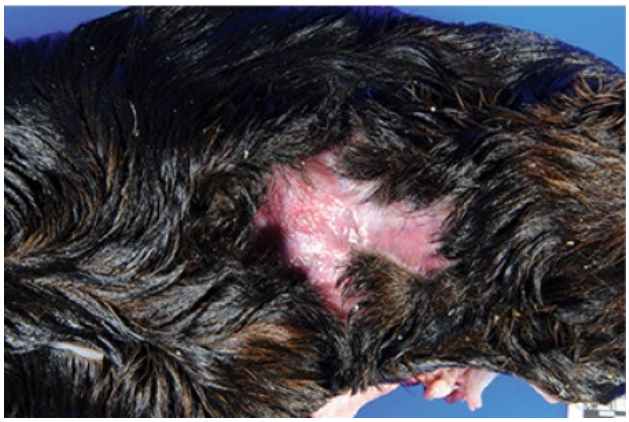


Figure 56.34 Spina bifida; failure of proper development of vertebra, spinal cord, and skin over lumbosacral area.



Figure 56.35 Hypospadia; failure of the ventral urethal fold to close from anus (far right) to prepuce (left).

Hypospadias are the failure of the urethral folds to join, and in severe forms may present as linear fissures from the perineum to the prepuce (Figure 56.35).

Epitheliogenesis imperfecta, an inherited absence of skin formation, presents as large red defects in the distal limb skin/hooves and additional oral lesions [89, 90].

Non-symmetrical skin defects can occur as a result of fragile skin collagen (asthenia/dermatosparaxis) or vesicobullous disease (epidermolysis bullosa, familial acantholysis of Angus calves, mechanobullous disease of Brangus calves). These conditions are hereditary and rare; definitive diagnosis requires histopathology and electron microscopy [87, 89].

Causes of Abortion

Embryonic Death

The early part of gestation is a time when fetal organ systems and extraembryonic membranes develop to achieve placentation, which is the point at which the chorion and endometrium attach. Prior to this, the uterus must be presented with

an embryo by days 15–17 in order to prevent luteolysis and maintain pregnancy [91]. Failure to do so leads to early embryonic death. Late embryonic death occurs after maternal recognition but prior to the fetal stage (days 42–260).

A variety of factors contribute to embryonic death, including heat stress, metritis, maternal endocrine abnormalities (progesterone), aged gametes, trauma, genetic abnormalities, nutritional deficiencies, and maternal–fetal incompatibilities [1]. Infectious disease is able to cause embryonic loss in three major ways: (i) the uterine environment of a febrile cow is also hyperthermic and may lead to loss; (ii) infectious organisms may directly infect the embryo or may interfere with the uterine environment; and (iii) endotoxin from Gram-negative bacterial infection may lead to premature luteolysis [1].

Fetal Death (Days 42–260)

Non-Infectious Causes of Abortion

Twinning is associated with higher rates (three to seven times normal) of stillbirth and abortion as well as retained membranes [92]. Abortion may occur at any time during gestation; rates of 30–40% have been reported for twin pregnancies [13]. In bovine twins, placental vessel anastomoses are almost always present, leading to fetal chimerism and freemartins.

Umbilical cord constriction is a rarely reported cause of abortion. This is largely due to three factors. Firstly, in the calf the umbilicus remains entirely within the amnion and is relatively short near term, such that rotation about the long axis (rolling over) is rarely possible. Secondly, the amnion is extensively fused to the chorioallantois, preventing rotation of the amnion within the chorioallantois, unlike the mare. Thirdly, at five to six months' gestation, the length of the calf exceeds the amnion width. By this stage, 95% of calves adopt an anterior presentation and cannot rotate around their transverse axes, preventing umbilical twisting and compression. In the mare, fetal anterior presentation is not firmly adopted until the ninth month [93]. If these assumptions are valid, then any constriction, twist, or entanglement should have occurred before the third trimester. Development would then proceed with relative normalcy until near term, when progressive umbilical constriction could impair venous and allantoic outflow. The result would be placental edema and possible abortion. Such a situation was suggested in a report of term abortion with placental edema in a Belgian White Blue calf, a breed in which it may be more common. Calves have had their cords wrapped around the abdomen, legs, or neck [94].

Abortion may be observed in association with severe abdominal trauma or stress, starvation, fetal hyperthermia or hypoxia, genetic abnormalities, and environmental causes (toxic plants, phytotoxins, mycotoxins, pharmaceuticals), and from iatrogenic intervention.

Toxic causes of abortion may be particularly difficult to diagnose, especially if they are not one of the better-known varieties. Specific lesions associated with certain toxins may be poorly described and therefore not easily recognized. Subclinical toxin exposure may lead to fetal death and abortion in an otherwise normal-appearing cow. Diagnosis in such cases is unlikely to be recognized by routine examination [20]. For specifics the reader is referred to Chapter 69 on the subject.

Therapeutic estrogenic compounds, PGs, or glucocorticoids may induce abortion [13]. Vitamin A is teratogenic, whether in deficiency or excess, and is associated with defective musculoskeletal and neural development which may lead to abortion [95].

Iodine deficiency has been reported in cases of fetal hyperplastic goiter in association with weak, alopecic, stillborn, and aborted calves [96]. Causes for hypothyroidism in cattle include forage/water low in iodine, selenium deficiency, ingestion of goitrogenic plants, or genetic reasons (Afrikaner cattle) [97, 98]. The thyroid gland in such cases is large and may weigh more than 30 g. Both iodine-deficient diets and high calcium intake have been implicated [99]. For suspected cases, removal of the entire thyroid gland is advised. Weigh the entire gland, fix half in formalin, and submit the rest fresh for iodine analysis. Gland weight above 30 g in addition to thyroid iodine concentrations below 1000 mg/kg (or thyroid iodine wet weight 0.03% or dry weight 0.01%) and consistent histological changes are necessary for diagnosis [98, 99].

Excessive dietary selenium has been associated with abortion or weak calves, with accumulation in the liver [100]. Conversely, selenium/vitamin A/E deficiency has been associated with poor fertility, cardiac mineralization, ascites, stillbirth, and abortion [101–104].

Finally, allergies or assumed anaphylactic reactions due to vaccines have been associated with abortion [13].

Infectious Causes of Abortion

The remainder of this chapter deals with contagious and non-contagious abortion-related pathogens. In Table 56.7 the approximate gestational stage of abortion is listed for various agents [105]. For each broad category of pathogen (bacteria, virus, protozoa, fungi), sequential preference is often given in order of perceived importance. Many pathogens are included in this section, partly due to their customary inclusion in other references concerning abortion, but

Table 56.7 Approximate stages of development when abortion occurs for specific pathogens.

Agent	Embryo	Early	Mid	Late
Anaplasma marginale				L
Trueperella pyogenes			L	L
Aspergillus fumigatus			L	L
Bluetongue virus		L	L	L
Bovine herpesvirus 1 (IBR)	V		V	V
Bovine viral diarrhea virus	V	V	V	
Brucella abortus				H
Campylobacter fetus	L		L	L
Chlamydia spp.				L
Coxiella burnetii				L
Epizootic bovine abortion			L	V
Histophilus somni				L
Leptospira spp.				V
Listeria monocytogenes				L
Mortierella wolfii			L	L
Mucor spp.			L	L
Neospora caninum			V	L
Salmonella spp.			V	V
Tritrichomonas foetus	L	L		
Ureaplasma spp.				L

Source: Adapted from [56, 105].
L, Low herd abortion rate; V, variable herd abortion rate; H, high herd abortion rate.

also for the sake of completeness and utility as a reference. Several of these organisms are discussed in other chapters and detailed information can be found therein. For those pathogens that are only mentioned in this chapter, further discussion is included in the following text.

Zoonotic pathogens include *B. abortus*, *Campylobacter* spp., *Mycobacterium bovis*, *L. monocytogenes*, *Coxiella burnetii*, *Leptospira* spp., and *Salmonella* spp.

Bacteria

Campylobacter fetus subsp. *venerealis* Infection

C. fetus is the cause of widespread venereal disease in cattle associated with infertility, embryonic mortality, and, uncommonly, abortion [106]. *C. fetus* is an obligate pathogen of the female reproductive tract, causing a mild transient endometritis [14]. After coitus, bacteria spread from the vagina to the uterus within two weeks. However, infection does not seem to impair fertilization and early embryonic development. Subsequent rapid bacterial replication in the uterus incites inflammation, leading to embryonic death between 30 and 70 days' gestation. This often occurs after maternal recognition of pregnancy, leading to prolonged estrus in cows. If bacterial replication in the uterus is slower, mid-gestation abortions are seen [14]. Non-venereally transmitted *C. fetus* subsp. *fetus* or *Campylobacter jejuni* infections are associated with sporadic abortion due to ingestion of infected material/tissues [106].

In infected herds, low fertility rates and abortion (<10%) are typical. Abortions are uncommon and occur most often at four to six months' gestation, but may occur in the last trimester; fetuses may be autolyzed, freshly dead, or even delivered alive [20, 27]. Gross fetal lesions are non-specific and include fibrinous pleuritis, pericarditis, and peritonitis [27, 107]. Membranes are not typically retained and may have lesions that resemble *Brucella* sp. infection, such as thickened leathery intercotyledonary spaces and variably necrotic cotyledons [19]. However, placental lesions are variable and often subtle or inapparent.

Darkfield examination of abomasal contents will reveal motile bacteria with rapid darting movements; this is characteristic of *C. fetus* or *C. jejuni* [14]. Organisms can be cultured from aborted tissues (lung, placenta, abomasal fluid), but isolation may be problematic as drying is highly detrimental to

viability. Several types of transport enrichment media are available; contact your local laboratory for preferred methods. Amies transport medium with or without charcoal is particularly useful for cultures [27, 106].

Leptospirosis

Leptospirosis is a worldwide infectious disease of many species (including humans), with highest prevalence in tropical areas [108]. Antigenically distinct serovars of *Leptospira* spp. (Icterohaemorrhagiae, Hardjo, Pomona, Grippotyphosa, etc.) may belong to more than one genomic species (*L. interrogans* sensu stricto and *Leptospira borgpetersenii*), since bacteria are classified into serogroups as well as genomic species [108, 109]. In cattle, *L. interrogans* serovar Hardjo type hardjoprajitno and *L. borgpetersenii* serovar Hardjo type hardjo-bovis are the most important causes of abortion, stillbirth, and reproductive failure [19]. These Hardjo types (hardjoprajitno and hardjo-bovis) are of particular importance in cattle; both are adapted/maintained in cattle (cattle are incidental hosts for Pomona and Grippotyphosa). Infection in maintenance hosts is characterized by efficient transmission, high incidence of infection, chronic disease, persistence in the urinary tract, poor antibody response, and few organisms in tissues [14]. The Hardjo strain important in the USA and found throughout the world is *L. borgpetersenii* serovar Hardjo type hardjobovis. The other, *L. interrogans* serovar Hardjo type hardjoprajitno, is a highly virulent organism found primarily in Europe [19]. The Pomona serovar is maintained in swine; cattle are incidental hosts. Serovar Pomona infections are associated with severe acute disease in adults, with hemolytic anemia, hemoglobinuria, mastitis, and abortion during the septicemic stage [110].

Bacteria are effectively shed in urine and may spread transplacentally, venereally, orally, via inhalation, and across conjunctiva [109]. Bacteremia follows a 4- to 10-day incubation phase, resulting in localization to the kidney and reproductive tracts. This in turn results in persistent shedding in urine and reproductive fluids. Maintenance of *Leptospira* spp. in the uterus may lead to fetal infection and abortion [14].

Pregnant cows generally abort in the last trimester or deliver weak infected calves; often abortion may be the only sign of infection in the herd [27, 109]. They may be sporadic or epidemic, occurring most often in the second and third trimesters [27, 111]. Abortion rates vary according to serotype; serovars Pomona and Grippotyphosa can approach 50%, while serovar Hardjo typically ranges from 3 to 10% [19]. Fetuses are often autolyzed and may be icteric [27]. In some cases the liver may have a green tinge due to cholestasis. Membranes may have intercotyledonary edema, and less commonly will have yellow/brown viscous fluid with diffuse cotyledonary necrosis (tan, uniformly affected) [26]. These changes are similar to those seen in BHV-1 infection.

Fresh specimens are essential for isolating the bacteria, as bacterial competition, desiccation, and acid pH are detrimental to leptospiral survival [109]. Pericardial fluid, abomasal fluid, and kidney are particularly good samples for finding bacteria. Bacterial lability and autolysis of samples makes isolation difficult and inefficient [27, 113]. Opportunistic bacteria (*T. pyogenes*, *Bacillus* spp.) may also be present in aborted specimens, obscuring leptospiral involvement [19].

One of the best methods for diagnosis is by serology using the microscopic agglutination test (MAT). Immunohistochemical tests are used for identifying bacteria in fixed tissues and fluorescent antibody (FA) tests may be used on frozen material. The kidney, lung, placenta, and adrenal glands are most useful for finding antigen [14, 19]. Refrigerated samples should be sent to the diagnostic laboratory promptly. PCR testing is available. For detailed discussion concerning diagnosis see Chapter 59.

Brucellosis

Brucella abortus is a highly contagious, zoonotic, intracellular bacterium that is an important cause of abortion and infertility in cattle. Cattle are the primary host of *B. abortus*, which is widespread in Africa, Asia, Europe, and Latin America [114]. In areas where cattle are housed with sheep or goats (western Asia, southern Europe), *Brucella melitensis* may infect cattle and cause abortions [115].

The cow is the source of infection, and the bacterium is mainly spread by placental tissue and uterine discharge, but spread via milk, contaminated feed, and in utero does occur. Uterine discharge may contain *B. abortus* for two weeks prior to and two to three weeks after calving or abortion [14]. Acute infection is characterized by abortion, delivery of weak or premature calves, retained fetal membranes, metritis, infertility, and sterility in cows and bulls [114]. Carpal hygromas can be common in certain areas and may be the only obvious sign of infection [115]. In managed vaccinated herds infection may be subclinical, whereas in fully susceptible herds abortion rates may be as high as 70% [116].

Bacteria penetrate the nasal/oral mucosa and incite a local or regional lymphadenitis, with enlarged lymph nodes that may be hemorrhagic. In time, bacteria spread hematogenously, with a bacteremia that may be recurrent and persistent. *B. abortus* has an affinity for the placental trophoblasts, in which they grow to large numbers, and is associated with persistent inflammation and necrosis. Infection then spreads to the fetus [19].

Abortion is the principal sign, occurring in the second half of gestation, after the fifth month. Infected cows may

abort only once, thereafter delivering term calves that may or may not be infected [116].

Placentitis is a consistent feature. Fetal membranes are often retained and contain yellow slimy exudate with gray/yellow flocculent debris between and especially surrounding the cotyledons [19]. Cotyledons are variably necrotic with thickening and fissures, or are soft to leathery, sometimes with sticky, brown, odorless exudate [19]. Intercotyledonary tissues may be similarly affected.

Fetuses are expelled one to three days after death; they are often autolyzed and may not have gross lesions [27, 116]. They may be edematous with serosanguineous cavity fluids and fibrinous pleuritis/pericarditis/peritonitis, pneumonia, and multiorgan hemorrhage [19, 114, 117]. Abomasal fluids may have yellow flocculent material [116]. Pneumonia in aborted calves is an important and outstanding feature; tissues are firm, sometimes presenting with a cobblestoned surface or small white foci [19, 116].

The gold standard for diagnosis is the isolation of *B. abortus* from the fetus or placenta (abomasal contents, spleen, lung), but this may be difficult. Serology is the best method to detect infection, using the milk ring test and card agglutination test. PCR methods are used to differentiate vaccine strains from wild-type bacteria. Organisms are plentiful in aborted tissues. Vaccination of pregnant cattle with strain RB51 is associated with abortion and should be avoided [115, 118]. For detailed discussion refer to Chapter 60.

Listeria Infection

L. monocytogenes and *Listeria ivanovii* are associated with disease in cattle, which takes the form of meningoencephalitis, neonatal septicemia, and abortion [119]. *Listeria* spp. are common in the environment, particularly in colder temperate climates. Disease in cattle is often associated with feeding spoiled silage where bacteria grow to large numbers. Hematogenous spread to the placenta 5–12 days later with subsequent fetal sepsis occurs. If infection occurs early in the last trimester, rapid fetal septicemia and death occurs. Abortion occurs a few days later, with partial masking of lesions due to autolysis [19]. Infections near term are associated with significant metritis and septicemia in the dam, with dystocia and retained membranes. In these cases, autolysis may be less severe, allowing better visualization of fetal lesions [19].

Abortions are sporadic and rarely exceed 15% [119]. They typically occur in the third trimester but may occur in the second. Fetuses die in utero and exhibit varying degrees of autolysis. Even if fetuses are well preserved, lesions may be few; the most commonly seen are pinpoint white/tan foci of liver necrosis that may mimic herpesvirus infection [120]. Small abomasal erosions, fibrinous serositis, pneumonia, and polyarthritis may also be seen [20, 51]. Placentas are often retained; changes include multifocal cotyledonary necrosis and intercotyledonary placentitis with gray/white to red/brown exudate [19, 119]. Aborting cows exhibit disease before, during, and after abortion, including weight loss, metritis, and septicemia [14, 19]. Recurrent abortion from year to year and in the same cow may be seen.

Organisms can be isolated from numerous fetal tissues; in some cases refrigerating specimens can aid in recovery of *Listeria* spp. [19]. Highly selective media are available. Gram staining of fetal fluids or tissue impressions reveal Gram-positive short rod bacteria in large numbers [14].

Histophilus somni (Formerly *Haemophilus somnus*) Infection

H. somni is a Gram-negative bacterial pathogen in cattle best known for causing pneumonia, arthritis, myocarditis, and thrombotic meningoencephalitis. It is a normal inhabitant of the male and female bovine genital tracts and is associated with infertility, abortion, and endometritis. Orchitis and epididymitis have been reported in bulls. Natural breeding spreads the bacterium. Transmission by AI is unlikely, as many strains are sensitive to the antimicrobials used to preserve frozen semen [19].

The association of *H. somni* with infertility is controversial; this bacterium is a normal inhabitant of the reproductive tract and research results are mixed [14]. As a cause of abortion, *H. somni* is not common; abortions are sporadic and infection rarely results in outbreaks. Typically, abortions occur in the second half of gestation. Placental lesions concentrate on cotyledons, where there is necrosis due to vasculitis and thrombosis. Such lesions are characteristic of this organism and indicate hematogenous spread from the vagina or respiratory tract. Fetal death is rapid and autolyzed carcasses are characteristic. Gross fetal lesions are uncommon; fibrinous bronchopneumonia may be seen [19].

Organisms may be cultured from abomasal contents and membranes. However, light bacterial growth from the placenta may be due to contamination from the birth canal. Culture must be interpreted in conjunction with necropsy findings [14].

Salmonella Infection

Salmonella spp. cause infectious and contagious disease in humans and livestock around the world. *S. enterica* subsp. *enterica* is divided into hundreds of serovars, of which Dublin is best associated with cattle abortion, although other serotypes may be involved, like *typhimurium* [121]. *Salmonella* Dublin is regionally distributed around the world and is known to persist on farms due to periodic fecal shedding, particularly around the time of parturition [121].

It is a cattle-adapted pathogen that causes enteric disease/dysentery, pneumonia, or polyarthritis in adults, but may also present with abortion as the principal sign [121]. Organisms survive in the environment for months to years and may be transported from farm to farm [122]. Bacterial infection in the dam leads to hematogenous spread to the placenta and subsequent placental failure and abortion, with or without fetal infection [19]. Abortion may be due to infection of the fetus, but as abortion may also occur in association with stressful events, pyrexia, and maternal enteritis may precipitate expulsion [19]. Abortions are typically sporadic to epizootic, occurring most often in the second half of gestation, often with retained membranes [121].

Aborted calves are often autolyzed and may be emphysematous. Pale foci of necrosis may be seen in the liver. Fetal fluids are fibrinous and amber colored; membranes are thickened and red/gray with yellow exudate and caruncular fragments on the cotyledons [19, 27]. Organisms can be recovered from placental tissues and abomasal contents [19].

Yersinia pseudotuberculosis Infection

This Gram-negative bacterium is associated with enterocolitis, caseous mesenteric lymphadenitis, and septicemia in calves. Apparently healthy cattle may carry the bacteria subclinically [123]. Sporadic abortion (often in the second half of gestation) has been reported, possibly due to uterine infection via transient bacteremia from the gut flora [124].

Fetuses typically have minimal autolysis; body cavities contain abundant fluid with fibrin tags, and livers may have multiple pale tan foci of necrosis [19, 27, 124]. Lungs may be firm due to bronchopneumonia [124, 125]. Microscopic lesions are characterized by abundant necrosis, vasculitis, and large bacterial colonies. Cotyledons are thickened, red/tan, with little exudate and caruncular tissue tags; intercotyledonary areas have fibrosis [19, 27]. Bacteria may be cultured from various tissues.

Trueperella (*Arcanobacterium*) *pyogenes* Infection

A common cause of suppurative inflammation in cattle that may act as a primary pathogen or a secondary invader, *Trueperella pyogenes* is a Gram-positive commensal organism in the respiratory, gastrointestinal, and urogenital tracts in healthy cattle. Tissue invasion leads to abscessation in any organ, causing a wide variety of clinical signs [126]. *T. pyogenes* is often isolated from aborted tissues and is considered to be a primary pathogen in cattle abortion [19].

Uterine infection occurs hematogenously or via ascending infection, which is often acquired in the postpartum period, in part due to retained membranes. Fetal infections are not consistently present. The ensuing endometritis is associated with discharge and pyometra, although the cow may appear normal [126].

Abortion can occur at any time throughout gestation, but the cow is often in the second half of gestation. Abortions are generally sporadic but may occur in clusters. Retained membranes are common; cotyledons are covered with yellow/brown exudate and intercotyledonary tissues are thick, opaque, and covered with similar exudate [27, 126]. Fetuses exhibit variable degrees of autolysis with fibrinous exudates on serosal surfaces; sometimes lungs are dark red with yellow foci or there is a characteristic hemorrhagic tracheal cast consistent with suppurative bronchopneumonia [19, 51] (see Figure 56.6).

T. pyogenes is readily isolated from fetal lung, abomasal contents, or membranes.

Various Opportunistic Bacterial Infections

Sporadic abortions in cattle may be caused by environmental and endogenous opportunistic bacteria, including *Bacillus* spp., *E. coli*, *Acinetobacter* spp., *Mannheimia haemolytica*, *Nocardia* spp., *Aliarcobacter* spp., *Pasteurella* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Helcococcus*, and *Serratia marcescens* [13, 127–129]. Almost any bacterium that can gain access to the dam's bloodstream has the potential to infect the conceptus.

Abortions occur most often in the second half of gestation. Fetal membrane retention and autolysis vary. Membranes are thickened with yellow/brown exudate (Figure 56.36). Fibrinous exudates may be found in body cavities. Fetal bronchopneumonia (white spotted lungs) is sometimes present [27].

Bacillus licheniformis is considered an important isolate associated with abortion, particularly in Europe. Outbreaks may be common. *B. licheniformis* apparently has little

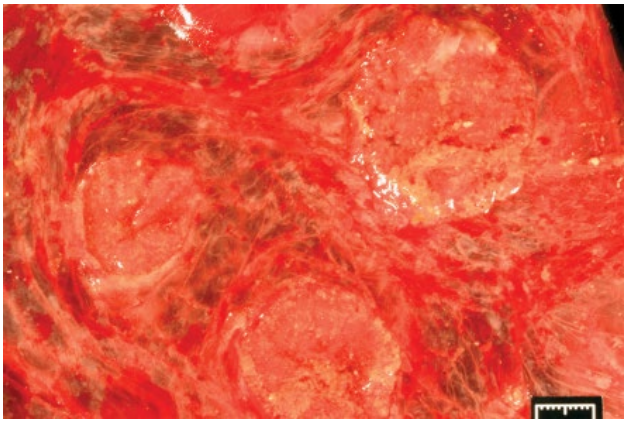


Figure 56.36 Chorioallantois with bacterial placentitis. Hemorrhage and fibrinous exudate cover the cotyledons and intercotyledonary spaces. *Source:* Photo courtesy of J. Edwards.

inherent pathogenic potential, and reports of disease often implicate coinfection, such as with *N. caninum* and BVDV [9, 130]. Fetal edema, fibrinous pericarditis, granulomatous hepatitis, bronchopneumonia, and placentitis have been reported [130]. Infected silage is implicated as a cause of cow infection, with a resulting bacteremia that may infect the cotyledons and spread to the fetus.

Cattle can develop subacute anthrax (*Bacillus anthracis*), with localized disease in the respiratory, gastrointestinal, and subcutaneous tissues. Pregnant cattle that survive infection may have fetoplacental infections as a result of bacteremia; gross fetoplacental lesions may be absent [131]. More severely affected cattle may present with protracted fever, subcutaneous edema, hemorrhagic vaginal discharge, an edematous fetus, bloody amniotic fluid, placentomal hemorrhage, and lack of typical lesions in the maternal liver, spleen, and kidneys [132, 133].

Mollicute Infection

The Mollicutes are bacteria that lack cell walls and have a preference for mucosal and serosal surfaces. They are known causes of chronic respiratory, urogenital, mammary, musculoskeletal, and ocular disease in ruminants. Coinfection with other pathogens is common. *Mycoplasmopsis* (prev. *Mycoplasma*), *Metamycoplasma* (prev. *Mycoplasma*), *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* spp. have been associated with male and female reproductive disease in cattle, including abortion [134]. These species, including those known to cause disease, are often found in the mucous membranes and reproductive tracts of normal animals.

Endometritis, granular vulvovaginitis, salpingitis, infertility, seminal vesiculitis, epididymitis, and granular balanoposthitis occur with infection. Transmission may occur venereally, mechanically (including AI as they can withstand freezing), and by respiratory routes [134]. Disease is multifactorial and associated with intensive husbandry.

Mycoplasmopsis (*Mycoplasma*) *bovis*, *Mycoplasmopsis* (*Mycoplasma*) *bovigenitalium*, *Metamycoplasma* (*Mycoplasma*) *canadense*, *Mycoplasma leachii* (type 7), and *U. diversum* have been reported causes of abortion in cattle, but *M. bovis* and *U. diversum* abortions are the best known and characterized [134–138]. The association of these organisms with abortion in general is controversial and is not well understood. However, *M. bovis* is not a normal inhabitant of the reproductive tract and thus its presence in an abortion is significant. Mycoplasmal abortion is uncommon. Reported lesions include placentitis, fetal bronchopneumonia, and myocarditis [14].

U. diversum is the most important abortion-related pathogen in this group, causing embryonic death, abortion (often in the last trimester), stillbirth, weak calves, and neonatal pneumonia [19, 134]. Outbreaks of *U. diversum* infertility have been reported lasting up to six months, with conception rates as low as 20% [14]. Fetal membrane retention is common. Since few diagnostic laboratories attempt to culture this organism, there is wide regional variability in diagnosis frequency. In the USA many cases are reported from Canada [27].

Grossly, the amnion is often severely affected with thickening/fibrosis, mineralization, necrosis, hemorrhage, fibrin, and meconium staining [19, 139]. The chorioallantois may be similarly affected with white to brown exudate. Aborted fetuses are commonly fresh [120]. Fetuses and newborn calves may have firm poorly aerated lungs. The constellation of amnion, chorioallantois, and lung lesions is characteristic of *U. diversum* infection [19].

Definitive diagnosis requires culture of organisms from stomach contents, placental tissues, liver, or lung in addition to consistent lesions [140]. These organisms preferentially localize to the fetal–maternal interface (placentome). Specific media are required for culture; consult the diagnostic laboratory for preferred methods. Samples should be refrigerated and transported immediately. Deliver samples in frozen media (Stuart broth, Amies without charcoal), preferably in liquid nitrogen or dry ice rather than wet ice [141]. PCR methods for *M. bovis* and general *Mycoplasma* spp. are available.

Chlamydia-Like Organism Infection

Bacteria of the order Chlamydiales are obligate intracellular parasites, ubiquitous, infect a wide range of vertebrate species, and cause a wide variety of diseases [142, 143]. *Chlamydia abortus*, *Chlamydia pecorum*, *Chlamydia psittaci*, *Waddlia* spp., and *Parachlamydia* spp. are of interest in cattle production. Abortion in cattle is usually caused by *C. abortus*, but *C. psittaci* abortion has been reported [144]. *Chlamydia* spp. have also been associated with mastitis and infertility [14]. *C. pecorum* is associated with enteritis, pneumonia, neurologic disease, polyarthritis, and conjunctivitis. Infectious forms may be shed in feces, urine, semen, uterine fluids, and ocular/nasal discharges [145]. Ingestion or inhalation of the organism leads to infection.

Parachlamydia spp. and *Waddlia chondrophila* have been isolated from aborted calves in Europe and in the USA [146–148]. The overall contribution of *Waddlia* and *Parachlamydia* spp. to cattle reproductive disease is uncertain.

C. abortus is an uncommonly diagnosed cause of abortion in cattle [27, 149, 150]. Fetal loss is more sporadic than in sheep, but abortion rates of up to 20% can occur [149]. Although the prevalence of occult chlamydial infections may be high in some areas, currently the contribution of such infections to fetal losses is unclear [149, 151, 152]. The method of natural transmission of infection in cattle has not been verified but is probably through contact with contaminated fetal fluids and tissues, as in sheep [153].

Affected cows may show little sign of disease, delivering aborted, stillborn, or weak calves, sometimes with retained membranes [154, 155]. *C. abortus* abortions typically occur during six to eight months of gestation, often presenting with necrotizing placentitis, with or without vasculitis [146]. Tissues have leathery, reddish, opaque intercotyledonary patches and multifocal cotyledonary necrosis. Fetuses may have pink/red subcutaneous edema, ascites, thymic and subcuticular petechiae, and serofibrinous pleuritis/peritonitis, which in some cases may be severe [154, 155]. Chronically infected calves may have nodular mottled livers and enlarged lymph nodes [155]. *Parachlamydia* and *Waddlia* related abortions also exhibit necrotizing placentitis, but vasculitis is uncommon [146]. Experimental *W. chondrophila* infection led to placentitis but not abortion [156].

Diagnosis of chlamydial abortion requires examination of the placenta, where organisms multiply in cotyledons [153]. Cotyledons and uterine discharges contain abundant organisms [14]. Isolation is the gold standard for diagnosis, which is necessary to identify species and strains/serotypes/subtypes; such methods require specialized media and special expertise [157]. It is important to recognize that many cattle harbor chlamydiae in their intestinal tracts, which may contaminate aborted tissues and confound complement fixation tests and PCR, leading to false-positive results [153]. Exposure of cows may be common and single titers are of questionable worth, although a rise in titer is suggestive of significant infection as delivery of an infected fetus leads to a rise in titer two to three weeks after parturition [14]. Specific antibody in fetal thoracic fluid or tissues is confirmative [19]. The presence of placentitis with identification of organisms within lesions by immunohistochemistry (IHC) as well as PCR is diagnostic. Placental smears stained with Giemsa, Gimenez, or modified Ziehl–Neelsen acid-fast methods are useful for diagnosis [19, 155]. Chlamydial organisms should be considered as serious zoonotic agents and may cause abortion in humans [158].

Epizootic Bovine Abortion (EBA)

EBA is a regionally important and specific disease confined to the western USA. It is associated with certain pine ranges that sustain the argasid tick *Ornithodoros coriaceus* [159]. The etiologic agent, *Pajaroellobacter abortibovis*, is transmitted by the tick [159, 160]. Disease is typified by premature calving and abortion in otherwise normal cattle [27]. The disease in the fetus is a chronic one, developing over three months or more prior to abortion. Abortions occur sporadically or in severe outbreaks, often in the last trimester. Fetuses are expelled fresh; retained membranes are not typical. Lesions include petechiae in the mouth (particularly the ventral tongue), enlarged peripheral lymph nodes, ascites, splenomegaly, dermatitis, large and coarsely nodular livers, and small thymuses surrounded by edema and hemorrhages [27, 159]. The presentation and lesions are characteristic. Diagnosis requires compatible lesions and herd history in addition to elimination of other causes [159].

Coxiella burnetii Infection

The cause of Q fever in humans, *C. burnetii* is a Gram-negative, obligate intracellular rod bacterium that replicates within macrophages [161]. Organisms are hardy and resist desiccation, pH change, ultraviolet radiation, and disinfectants. Found worldwide except in New Zealand, *C. burnetii* is present in a wide variety of rodents, birds, wild and domestic animals (including reptiles, dogs, cats, pigs, horses), ticks, and domestic ruminants, the latter serving as reservoirs for human infections [161]. Prevalence is variable worldwide; in the USA prevalence in 316 bulk milk samples was greater than 90% [162]. Routine pasteurization kills the organism [110].

In endemic areas infected calves may clear the infection or become latently infected. During late pregnancy hormonal changes are thought to trigger *C. burnetii* replication in the uterus, fetus, and mammary gland [161]. Infected sheep, goats, and cattle are typically asymptomatic when they present for late-term abortion, stillbirths, and weak neonates [161, 163]. Abortion in cattle is uncommon to rare. The organisms are shed in feces, milk, urine, fetal fluids, and fetal membranes in large numbers [161]. Infection usually occurs through inhalation of infected aerosols, but spread through infected equipment, manure, and ticks is possible.

Approximately half of seropositive heifers shed *C. burnetii* at calving; there is reduced shedding with age. Chronically infected cows may shed organisms in milk, feces, and vaginal mucus intermittently for several months to years [164]. Gross lesions are confined to the placenta, where the intercotyledonary zones are covered by abundant, white, inspissated exudate, giving the tissue a thick leathery appearance [19]. Multifocal mineralization and pericotyledonary necrosis may develop [161].

Placental exudate smears stained with Gimenez or Ziehl–Neelsen acid-fast staining methods show numerous organisms, but these may be easily confused with *Chlamydia* spp. PCR and enzyme-linked immunosorbent assay (ELISA) are effective in diagnosis. The mere presence of *C. burnetii* in aborted tissues is not sufficient evidence for diagnosis; cows may carry the organism long term and they may be in placentas of subsequent pregnancies [19, 164].

Tuberculosis

Mycobacterium tuberculosis (syn. *M. bovis* and *Mycobacterium caprae*) has beenlargely eliminated from many countries around the world, yet remains enzootically or in limited

geographic areas in Central and South America, North America, Africa, Europe, Asia, and Oceania. Disease caused by *M. caprae* is not significantly different from that caused by *M. bovis*. *Mycobacterium* infection occurs in a wide range of hosts, including domestic livestock, cats, and dogs. The bacterium is a major economic problem in terms of trade and industry as well as being a zoonotic pathogen. One of the major problems encountered in terms of eradication is the prevalence of *M. tuberculosis* in wildlife reservoirs such as farmed and wild cervids, European badgers, buffalo, bison, brushtail possums, and European wild boars [165, 166].

Mycobacterium is typically spread by inhalation or ingestion. With reference to theriogenology, bull carriers may spread infection through coitus [13]. An initial site of infection may develop into a persistent focus of bacterial replication, with subsequent hematogenous spread to various organs. Disease is characterized by the presence of tubercles, which are discrete, pale tan granulomas of various sizes in virtually any organ. They may resemble abscesses, with abundant caseous necrosis. In adult cattle, tubercles are most common in the lungs and the lymph nodes of the mesentery, pharynx, and thorax; generalized lesions are uncommon. *Mycobacterium* reaches the uterus hematogenously or by peritoneal spread to the uterine tubes. Uterine infection may primarily affect the serosa/muscularis or may concentrate in the endometrium. The uterine tubes may be severely affected [167]. Abortion due to endometritis often occurs late in gestation [168]. Retained placentas and purulent to caseous uterine exudate may be seen [13].

Congenital tuberculosis occurs in about 1% of at-risk calves [168]. Transplacental transmission may occur hematogenously via tuberculous endometritis with umbilical spread to the fetal liver (hepatic primary complex) and lymph nodes [166]. Alternatively, fetal swallowing or inhalation of tuberculous amniotic fluid may result in enteric or pulmonary lesions, respectively [168]. *Mycobacterium avium* has been reported as a cause of abortion in cattle housed with infected chickens [13].

At least 10 g of fat-free affected tissue should be submitted for culture.

Anaplasma Infection

There are currently three species of anaplasmal bacteria that infect cattle: *Anaplasma marginale*, *Anaplasma centrale*, and *Anaplasma phagocytophilum* (previously *Ehrlichia phagocytophila*, *Ehrlichia equi*, and human granulocytic ehrlichiosis agent) [169]. *A. marginale* is the cause of anaplasmosis in cattle and can infect buffalo, bison, deer, and other hoofstock. This infectious but non-contagious agent is spread by arthropod bites, mechanical means, or transplacentally [169]. Anaplasmosis occurs in tropical and subtropical areas worldwide. *A. marginale* infects erythrocytes, leading to extravascular hemolysis, anemia, icterus, fever, and weakness [170]. Initial infections are the most severe, leading to chronic persistent infection that is often subclinical. *A. centrale* is a milder pathogen, usually employed as an immunizing agent against *A. marginale* [171].

A. marginale is rarely implicated as a cause of abortion. Suggested causes of fetal loss include pyrexia, severe anemia, or stress in the dam. Aborted calves show variable liver and lung petechiation with splenic enlargement [172].

A. phagocytophilum is the cause of pasture fever in cattle and tick-borne fever of sheep and goats in northern Europe, although the bacterium is distributed worldwide. While currently considered to be one species, *A. phagocytophilum* strains causing ruminant disease differ in distribution, disease, severity, and target hosts from those causing human granulocytic ehrlichiosis [173]. The organism infects circulating granulocytes and large lymphocytes, which are best seen during clinical illness [171]. Signs include fever, cough, nasal discharge, and leukopenia [170]. Although present in the USA, cases of clinical illness in cattle have not been reported. *A. phagocytophilum* has been known to cause abortions (and storms) during first exposure to infected ticks during late pregnancy [9, 173].

Diagnosis of *Anaplasma* spp. can be made on Giemsa-stained blood smears during the acute phase in cattle, but it is not useful for carrier states. Blood from live cattle should be collected in anticoagulant and refrigerated to be sent to a diagnostic laboratory. Smears from the liver, kidney, heart, lungs, or peripheral blood vessels can be made from dead animals and air dried for staining [174]. Serologic tests and PCR methods are available.

Viruses

Herpesvirus

Bovine Herpesvirus 1 (BHV-1, Infectious Bovine Rhinotracheitis Virus, Infectious Pustular Culvovaginitis Virus) Infection BHV-1 virus is an important worldwide alpha-herpesvirus pathogen of cattle, known to cause abortion, genital disease, respiratory disease, encephalomyelitis, and severe systemic disease in neonates [27]. Other ruminants, including deer, pigs, goats, wildebeest, and buffalo are susceptible [175]. Infected cattle shed virus from oculonasal and reproductive fluids. Like other herpesviruses, latency is established after infection through the genital or respiratory tract [175]. Respiratory infection in naive pregnant females is likely to lead to fetal infection and abortion [176]. Clinical signs in adults are generally mild, with conjunctivitis or respiratory tract disease. However, signs may be absent at the time of abortion, which typically

occurs several weeks after initial infection [27, 175]. Abortion is typically seen in the second half of gestation, anywhere from two weeks to two to three months after clinical disease; fetuses are expelled three to seven days after death [13, 175]. Abortions may be sporadic in herds with previous exposure, which includes modified live vaccines [176]. If naive pregnant cattle are exposed, storms affecting 25–60% may occur [27, 177].

The virus spreads hematogenously in the dam to the caruncle, where it invades the placenta and then the fetus. Herpesviruses induce widespread cell necrosis in multiple organs, leading to rapid fetal death [19]. Fetuses show advanced autolysis with few lesions; tissues are red due to hemoglobin staining and abundant reddish fluid may be present in body cavities [175]. Pinpoint white foci of hepatic necrosis may be present [27]. Less commonly, pulmonary and renal hemorrhage with necrosis is seen [19, 178]. Renal necrosis may be pronounced [179]. Diffuse placentitis similar to that seen in leptospirosis may be found and yellow/brown amniotic fluid may be evident.

Fetal lung, liver, spleen, kidney, adrenal gland, and placenta are good sources of virus; samples should be kept cold. Freezing at −70 °C is acceptable, but standard freezing (−20 °C) will inactivate the virus [175]. Histopathology may give a presumptive diagnosis, which can be confirmed by IHC, PCR, FA detection, immunoperoxidase tests, or virus isolation. Kidney and the adrenal gland are useful for FA testing [27]. Cows that abort typically have low titers at the time of abortion and fetuses die so rapidly that fetal antibody production may be poor or non-existent [19].

Bovine Herpesvirus 4 (BHV-4) Infection A widespread gammaherpesvirus in cattle considered minimally virulent or avirulent in adults, BHV-4 may be an important cause of abortion [177, 180]. Presence of the virus in abortions is often associated with other pathogens [19]. Associated disease includes pneumonia, keratoconjunctivitis, orchitis, sudden death in neonates, digital dermatitis, mastitis, encephalitis, diarrhea, vulvovaginitis, postpartum metritis, and abortion [177, 181]. The contribution of BHV-4 as a cattle pathogen at this point is mostly hypothetical, with the exception of the latter three conditions [180]. The role of BHV-4 in abortion is incompletely understood [182]. Abortion is reported to occur between five and nine months' gestation. Descriptions of lesions in the literature are few, with no gross fetal or placental lesions reported. Cytomegalic intranuclear inclusions are seen microscopically in multiple organs [183].

Bovine Herpesvirus 5 (BHV-5) Infection A pathogen typically associated with severe meningoencephalitis in young calves, BHV-5 outbreaks are seen in South America and sporadically in Australia, Europe, and the USA (Figure 56.37) [184]. Transmission may occur through the genital tract, with evidence suggesting possible negative embryological effects [185–187]. The virus has been isolated from an aborted fetus [188].

Bovine Viral Diarrhea Virus (BVDV) Infection BVDV is one of the most important infectious causes of reproductive loss in cattle worldwide. It is a pestivirus that uses the reproductive system for spread within herds. Negative effects on herd reproduction range from genital tract inflammation, reduced reproductive efficiency, and embryonic death to abortion storms, delivery of small calves, persistently infected calves, and congenital defects [19, 111].

Transplacental infection is likely in cattle [176]. Viremia leads to fetal infection as it is a primary target for the virus [189]. The manifestations of disease in pregnancy vary according to virus strain and stage of fetal development at the time of infection [176]. Fetal infection in the first and second trimesters often leads to embryonic death, resorption, mummification, or abortion [27]. Fetal calves infected with non-cytopathic viral strains between one and four months become persistently infected, immunotolerant calves that produce no antibody to the virus [111]. Those infected after four months may successfully eliminate the virus, but in some cases those infected between 100 and 150 days develop congenital anomalies [27, 111]. Such anomalies are quite variable but include central nervous system anomalies (hydranencephaly, hydrocephalus, cerebellar hypoplasia, microphthalmia, retinal dysplasia, cataracts), thymic hypoplasia, hypotrichosis, bone defects, brachygnathism, arthrogryposis, and pulmonary or renal hypoplasia/dysplasia (Figure 56.38). Necrotizing myocarditis leading to chronic passive hepatic congestion and

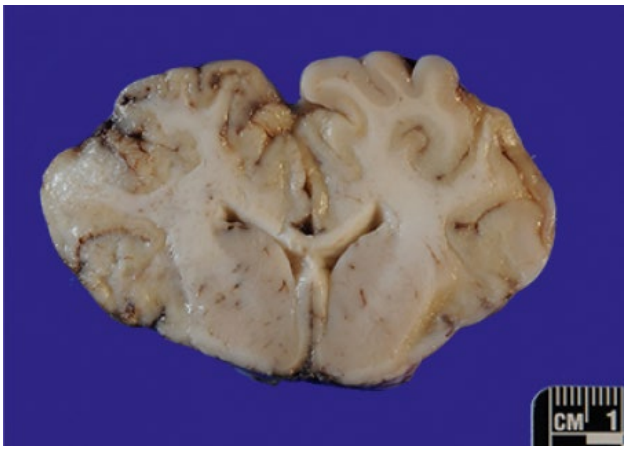


Figure 56.37 Brain, coronal section. BHV-5 necrotizing meningoencephalitis; severe tissue loss over the left cerebral gryi.

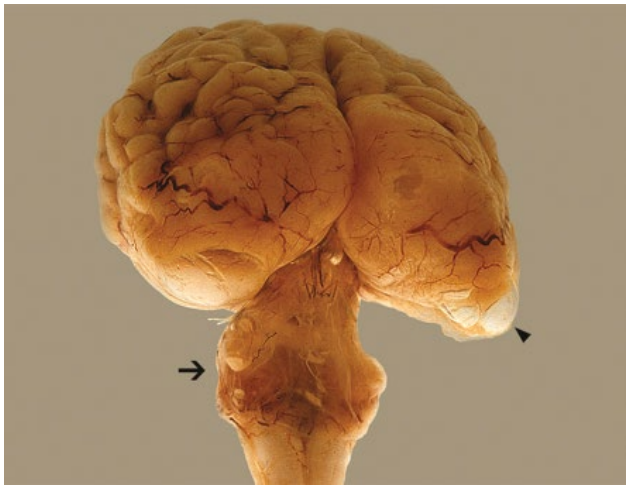


Figure 56.38 Brain, dorsolateral aspect. Hydranencephaly and cerebellar hypoplasia, BVDV in utero infection. The caudal aspect of the cerebrum lacks gyri and is thin to the point of translucency (arrowhead). The cerebellum is vestigial (arrow). *Source:* Photo courtesy of J. Cooley.

fibrosis with ascites as well as congenital diabetes mellitus have been reported (see Figure 56.9) [27, 190]. Individual herds may have only a few calves with congenital defects; consecutive calves may have the same defects [191]. More often, fully developed but small (50–66% normal size) calves are delivered [192]. Infections in the last trimester do not typically produce disease as calves are immunocompetent and produce anti-BVDV antibodies [189].

Fetal death and abortion typically occur in the first trimester, but can occur at any time. The abortion rate varies but is usually low [189]. There is a delay between fetal death and expulsion (as much as 50 days), which often produces autolyzed specimens from which it is difficult to detect the virus. Additionally, dams often seroconvert during this delay, making serum titers of little value [113]. Lesions aside from teratogenic changes are therefore difficult to appreciate and rarely noted. Enlarged spleens, lymph nodes, and nodular mottled livers may be seen [189]. Thrombocytopenia inducing strains (types 1b and 2) may cause widespread petechiation and hemorrhage [193]. Placental lesions are generally not seen [51].

The virus is fairly resistant and can survive in moist tissues for several days; preferred samples for virus isolation include lymphoid organs [189]. Various other diagnostic methods are used including IHC, FA, and ELISA [27]. BVDV is widespread, and molecular evidence of infection in an aborted fetus does not prove it to be the etiologic agent. Herd history and fetal lesions consistent with BVDV infection are necessary for diagnosis [27]. A history of ataxic, small, or blind calves in the herd is suggestive. Fresh specimens and often multiple fetuses are required before the virus can be identified [113]. Evidence for transplacental transmission includes detection of BVDV in fetal tissues or precolostral antibodies in fetal serum or fetal fluids. However, a persistently infected (non-cytopathic BVDV infection) fetus will not produce antibody. Infected dams that carry persistently infected calves may have high titers that continue to increase until delivery [194]. Contaminated modified live BVDV vaccines have been reported to cause disease and congenital defects [176].

Recently, another pestivirus (HoBi-like, BVDV-3) has been associated with abortion and respiratory disease in cattle in Brazil, Southeast Asia, and Italy [195–197]. The origins of this virus are poorly understood; hypotheses include viral emergence from South America or transmission from water buffalo to cattle. Natural infection has not been reported in North America, Europe, Australia, India, and Africa so far [195].

In natural cases, seromucous oculonasal discharge, tracheitis, bronchopneumonia, and gastroenteritis have been reported in infected calves. Abortions were seen in the second trimester [198]. Experimentally, hyperthermia, oculonasal discharge, and lymphopenia were seen in calves. Lambs also exhibit nasal discharge, while pigs show no signs but will seroconvert. Peak seroconversion occurs in calves by 21 days after infection; nasal and fecal shedding occur [195]. HoBi-like virus has been isolated from bull semen. In utero infection may produce persistently infected calves, similar to BVDV. Current testing is complicated by the similarity of the HoBi-like viruses to BVDV.

Cattle are susceptible to border disease virus (BDV) infection, which is a pestivirus generally associated with small ruminants. The virus can be transmitted between sheep and cattle, and abortions have been reported in association with infection. Infected cattle have signs similar to those of BVDV infection; they may develop diarrhea, and persistent infection in calves can occur [199]. Experimental infection caused growth retardation and hydrocephalus in calves [200]. Many antigen and antibody tests cannot discriminate between BVDV and BDV; specific PCR assays, with or without sequencing, are necessary for diagnosis.

Orbivirus

Bluetongue Virus (BTV) Infection

BTV is the cause of a widespread arthropod-borne orbiviral disease of domestic and wild ruminants, particularly of sheep [201]. Virus distribution is confined to areas where susceptible hosts (wild game, sheep, and cattle) are found and the *Culicoides* spp. biting midges are present for transmission [201]. BTV is generally considered non-contagious and infected animals shed little virus, except possibly in the semen of viremic bulls [19]. However, novel serotypes with

minimal virulence can be horizontally transmitted among sheep and goats [202]. BTV infection may be seasonal, depending on the climate and numbers of *Culicoides*; in temperate zones, late summer/early autumn is common [203]. The range of infection has increased since 2000, as a result of additional *Culicoides* spp. vectors, emergence of novel BTV serotypes, and changes in climate [202]. After infection, animals may be viremic for several weeks, but this does not lead to immunotolerance or persistent infection [176, 204]. Clinical signs in adult cattle are rare and mild, particularly in enzootic areas [177]. When clinical disease occurs, signs include the formation of ulcers in the mouth and tongue as well as on the muzzle. The coronary bands develop hyperemia and ulceration, and hoofs may slough [191].

After introduction into the skin, the virus is transported to local lymph nodes where it replicates. Subsequent hematogenous spread to secondary organs then occurs. BTV replicates in macrophages, lymphocytes, and vascular endothelial cells, causing host cell death. This results in widespread injury to small blood vessels which leads to vascular leakage, edema, subsequent thrombosis, and tissue infarction [205].

Infections during pregnancy cause congenital abnormalities more commonly than abortion; early embryonic death, mummification, and stillbirth also occur [19]. Congenital abnormalities due to BTV are sporadic and are known to occur principally in the USA and South Africa, where modified live vaccines are used. These vaccine strains were considered largely responsible for fetal malformations [206]. Abortions associated with BTV2 and 9 vaccines have been reported in Europe [207]. Until recently, transplacental transmission was thought to occur only in laboratory modified strains, although now serotype 8 (BTV8) in Europe has been shown to frequently cross the placenta [27, 208, 209]. BTV8 is unusual not only for its virulence in cattle, but also for its wild-type transplacental transmission and oral transmission in field and experimental reports [210–212].

Like BVDV, fetal disease is associated with gestational age at infection. Fetal infection prior to day 70 may lead to fetal death and absorption, while infection between 70 and 130 days can cause stillbirth, weak calves, hydranencephaly, or abortion. If infection is just prior to fetal immunocompetence, hydrocephalus or porencephaly may result. Fetal infection after 150 days can cause encephalitis or premature birth, but malformations do not occur [27, 206]. Abortions occurring in the last trimester have been associated with hydranencephaly [213].

Isolation of BTV is difficult and the virus is inactivated by freezing at −20 °C [176]. Virus may be isolated from blood (in anticoagulant: EDTA, heparin), spleen, or lymph nodes if tissues are sent refrigerated to an appropriate laboratory [204]. PCR techniques use whole blood in EDTA. Live attenuated and killed vaccines are available. Live vaccines are effective but are associated with abortion and congenital anomalies when used in pregnant females in the first period of pregnancy [204]. Fetal BTV antibody from serum or cavity fluids indicates in utero exposure [27]. Complementary lesions are needed for diagnosis.

Epizootic Hemorrhagic Disease Virus (EHDV) Infection

EHDV is an orbivirus transmitted by *Culicoides* midges that affects wild and domestic ruminants, particularly white-tailed deer [214]. Of the many serotypes found worldwide, Ibaraki virus, an Asian strain of EHDV serotype 2, has long been considered the only strain of significance to cattle. However, recent outbreaks of severe disease in cattle have been seen with serotypes 6 and 7 in Turkey and Israel [215, 216]. Disease in cattle mimics BTV; mortality is often low and morbidity varies [214]. Abortions and stillbirths have been reported in outbreaks of Ibaraki virus infection in Japan [217, 218].

Palyam Serogroup Orbivirus Infection

This group includes at least 15 viruses that are primarily found in Africa, Asia, and Australia and are associated with abortion and teratology in cattle [219]. *Culicoides* midges are natural vectors, but the virus has also been isolated from mosquitoes and ticks. Of these, the Chuzan (Kasba) virus is best known, causing hydranencephaly and cerebellar hypoplasia in calves [220, 221]. The presence of infection in herds is usually found after abortion and malformations are seen. Abortions may occur at any stage of gestation, being sporadic or epidemic [219]. Antibodies in precolostral serum are helpful in establishing a diagnosis [219].

Bunyaviridae

Rift Valley Fever Phlebovirus (RVF) Infection

RVF is the cause of severe disease in domestic ruminants and humans; the virus is largely confined to sub-Saharan Africa and Madagascar [222]. Mosquitoes are the main route of transmission, with many capable species, including some found in the USA [19, 222]. Disease is most severe in young ruminants. In calves, severe hemorrhagic diarrhea and liver necrosis (with icterus) develop over a rapid course, with mortalities ranging from 10 to 70%. Adult cattle may show only abortion, which can occur at any gestational stage and may reach 80–90% [177, 222]. Abortion is due to fetal death; specimens are autolyzed and exhibit liver necrosis [223]. Fetal livers are enlarged, soft, friable, and yellow/red with hemorrhages or subcapsular hematomas [224]. Placental lesions have not been described. Aborted tissues

contain large amounts of virus [19]. Hepatic lesions are considered diagnostic; differentials include BTV and Wesselsbron virus. The virus may be isolated from the liver, spleen, and brain; keep refrigerated or freeze and send on dry ice. Serological tests are available [225].

Akabane and Simbu Serogroup Orthobunyavirus Infection

This group includes about 25 viruses in the *Bunyavirus* genus that occur worldwide, including the Akabane, Aino, Tinaroo, Peaton, Shamonda, Shuni, and Schmallenberg viruses (SBVs) [226–230]. The Akabane virus, which occurs in Australia, Japan, China, Korea, the Middle East, and Kenya, is possibly the most important pathogen of this group, although SBV is now on the rise in Europe [19, 231]. Transmission is via *Culicoides* gnats and probably mosquitoes [230, 232]. Clinical disease in adult cattle may be mild or inapparent, with reduced milk yield, diarrhea, or inappetence [230, 233]. Clinical disease is more characteristic of SBV than in other viruses of this group [234]. Certain variants (genogroup Ia, Iriki strain) of Akabane virus are known to cause neurologic disease outbreaks (bovine epizootic encephalomyelitis) in calves and adult cattle in East Asia [191]. The Shuni virus has recently emerged as a cause of neurologic disease in calves and abortion in Israel [235].

Infection during pregnancy may produce abortions, malformations, mummies, and stillbirths [19]. Abortions occur in the third trimester and can occur as clusters or epidemics [230, 232]. Viruses in this group are neurotropic and are known to cause arthrogryposis, muscular hypoplasia, and hydranencephaly defects in domestic ruminants [233, 236–238]. Hydrocephalus, cerebellar hypoplasia, porencephaly, scoliosis, and cardiopulmonary defects are also reported [177, 233]. The gestational stage at which infection occurs determines the severity and outcome of infection, with decreased severity seen in later infections [230, 232].

SBV was first noticed in the summer of 2011 in northern Germany and the Netherlands as a febrile syndrome in adult cattle; signs included fever, decreased milk production, and diarrhea [234]. SBV is the first outbreak of a Simbu serogroup virus in Europe and has now spread across much of Western Europe [239]. The virus is associated with abortion and congenital malformations in cattle, sheep, and goats. The losses in cattle due to decreased milk production and return to service may be more costly than losses due to malformed calves, which in the Netherlands' outbreak occurred in 1–3% of farms [239].

SBV antibody seroprevalence was 72% in cattle from the Netherlands between November 2011 and February 2012; a high seroprevalence in red deer, roe deer, and mouflon was also found. Current evidence suggests that biting midges (*Culicoides* spp.) are involved in horizontal transmission, but the details of horizontal transmission and its significance are poorly understood [239, 240]. SBV has been found to be shed in semen, unlike Akabane virus, and vertical transmission occurs [241]. The susceptibility of non-ruminant species to SBV is unclear [239]. SBV is unlikely to pose a risk to humans [242].

Virus can be isolated from blood and various fetal tissues and fluids [19]. PCR and immunochemical methods are available for detection in tissues. For SBV, brainstem tissue for PCR and pleural fluids for antibody testing have been reported as an optimal combination for diagnosis [243]. Differentials for arthrogryposis/hydranencephaly include BTV, BVDV, Chuzan, Wesselsbron, SBV, genetic causes, and toxins such as lupines.

Wesselsbron virus infection is caused by a mosquito-borne flavivirus primarily found in Africa. Disease is most severe in young small ruminants. Infection in adult cattle is typically subclinical [244]. Pregnant cattle may sporadically abort, deliver weak calves, or deliver apparently healthy calves. Certain viral strains can produce encephalitis, porencephaly, and cerebellar hypoplasia [19].

Bovine Parvovirus (BPV) Infection

Bovine parvovirus (BPV) is widespread in the USA and worldwide. Often associated with diarrhea in calves, it is an uncommonly diagnosed cause of abortion [19, 192, 245]. Virus can be transplacentally transmitted to developing fetuses [246]. Experimental data indicate first and second trimester fetuses are most susceptible [246]. If infected between days 107 and 150, fetal cerebellar hypoplasia may be seen [19]. Fetuses infected in the last trimester can respond immunologically, preventing death. BPV should be considered in herds with abortion and repeat breeding problems [245].

BPV is present in multiple fetal visceral tissues and in membranes [19, 246]. BPV can be grown in cell cultures, but care should be taken that supplemental sera do not contain BPV antibodies. Serum from fetuses infected in the third trimester will have specific antibody to BPV [247].

Miscellaneous Viruses

Bovine enterovirus (picornavirus), adenovirus, pseudorabies virus (Suid herpesvirus 1), parainfluenza virus 3 (PI3), lumpy skin capripoxvirus, malignant catarrhal fever (gammaherpesvirus), foot and mouth disease virus (picornavirus), and bovine leukosis virus have been associated with abortion [13, 192].

Bovine PI3, generally considered a common mild respiratory pathogen, has been diagnosed uncommonly in abortions. Abortion calves have red rubbery lungs [248]. Prevalence in herds is high, and association with abortion may be incidental or contributory with other pathogens. Experimental infection

may cause bronchiolitis and interstitial pneumonia. Abortion due to PI3 is rare [27, 249].

Fetal antibodies to bovine enterovirus have been detected in high percentages of aborted fetuses tested in two studies [250, 251]. This virus has been associated rarely with significant enteric disease in adult cattle, and experimental reproduction of illness in calves has been difficult [252, 253].

Protozoa

Neospora caninum Infection

N. caninum is a common worldwide infectious apicomplexan parasite of domesticated livestock, dogs, and some species of deer [254]. Dogs (including coyotes, dingoes, and gray wolves) are definitive and intermediate hosts. Currently there is no evidence for infection in foxes [255]. Livestock are intermediate hosts that harbor tachyzoites that form tissue cysts [256–258]. Tachyzoites may be found in a variety of cell types, but tissue cysts are almost exclusively found in the nervous tissues, including brain, spinal cord, eye, and nerves. Infection occurs in both beef and dairy herds and it is a major cause of dairy cattle abortion in the USA and the UK [259–261]. Vertical transmission is a common and highly efficient route of infection in ruminants and can progress through multiple generations in congenitally infected heifers [256, 262]. Cattle may also become infected by ingestion of sporulated oocysts from canid feces.

Maternal seroprevalence is highly variable; seropositive cattle are more likely to abort than seronegative cattle [27, 263]. Antibody titers in infected cows may fluctuate or decline over time and many may be persistently infected, making interpretation of sample results difficult [27]. Serum samples from precolostral calves are useful for determining infection in suspect cows, due to the efficiency of transplacental transmission [264]. For abortions, specific antibodies in fetal serum (after 100 days' gestation) or brain/spinal cord tissue are indicative of infection but do not prove *N. caninum* as the abortigenic agent. Similarly, negative titers do not rule out neosporosis since fetal antibody formation depends on exposure level, gestational age, and duration of infection [27, 254, 265, 266].

Abortion is the only clinical sign in cattle, occurring from three months' gestation to term, but predominating at five to six months, a hallmark pattern of this pathogen [256, 259, 267, 268]. Abortions may be sporadic, endemic, or epidemic [269]. Calves may be stillborn, mummified, malformed, or born alive; calves may rarely show neurologic signs [73, 254]. They may also be small for their age, ataxic, or have flexion/hyperextension of the limbs [19]. In most cases of infection, asymptomatic congenitally infected calves are produced [267].

For definitive diagnosis an examination of the fetus is necessary. Fetuses are typically autolyzed with serosanguineous fluid accumulation. No consistent gross lesions are seen, although pale streaking in skeletal or cardiac muscle may be present [267]. Cotyledons may be necrotic and areas of malacia (soft, pitted, darker areas) may be seen in the brain or brainstem [19]. Submitting the entire fetus with placenta is optimal. Fetal brain is the most consistently affected tissue for finding lesions. IHC is highly useful for diagnosis since organisms may be few and difficult to find in autolyzed tissues [254]. The results of such methods should be viewed cautiously, as fetuses infected later in gestation may only have a mild *N. caninum* infection but die from a secondary pathogen [27]. Congenitally infected calves often have high precolostral titers, but antibody levels vary and may even be undetectable [9, 19].

Tritrichomoniasis

This is a worldwide venereal disease of bull-bred cattle due to *T. foetus* parasitism. Commercial AI services in the USA often screen for this pathogen, although custom-collected semen may be contaminated. This flagellated protozoan is an obligatory inhabitant of male and female genital tracts, occurring in both beef and dairy herds. Concurrent infection with *C. fetus* subsp. *venerealis* may be common. Females of all ages exhibit equal susceptibility and present with a history of bull-bred infertility due to embryonic death.

Infection typically causes vaginitis that may progress to endometritis; such infections may cause embryonic death and abortion (5 to >20%) in the first half of gestation, with uncommon later-stage abortions. The majority of pregnancies are probably lost by day 17. Fetuses aborted in mid pregnancy may be macerated and expelled with membranes. Cows may develop pyometra due to retained fetuses, which may present as multiple pyometras in a bull-bred herd. Uterine fluids are copious with little odor and can vary from watery and flocculent to red/brown and inspissated. Embryonic death after recognition of pregnancy (17 days) can lead to irregular cycling and return to estrus 60–90 days after breeding.

Placentas show little evidence of pathology aside from edema or the presence of white flocculent material and mild cotyledonary hemorrhage. Fetuses present in varying stages of autolysis, from fresh to macerated, and in many cases show no gross pathologic changes. Mild rumenitis with plaque formation can be seen occasionally [107]. In late-stage abortions bronchopneumonia and necrotizing enteritis have been seen.

Diagnosis depends on finding organisms in fresh fetal fluids/abomasal contents and pyometra fluids (directly from the uterus) where they are often plentiful. Tissues older than 48 hours are poor specimens. Commercial media are preferred for transport and culture (In-Pouch TF Bovine, Biomed Diagnostics, OR, USA) and samples should be

maintained between 22 and 37 °C. Buffered saline solutions (physiologic saline, lactated Ringer's) can be used, but samples should be kept cool (not frozen) and delivered as soon as possible (<48 hours) to a diagnostic laboratory. Positive identification of *T. foetus* may be complicated by other flagellated protozoan species. Immunohistochemical methods are also available to detect organisms in fixed tissues.

T. foetus infections may mimic *C. fetus* subsp. *venerealis* infections [9, 13, 19, 27, 269–272].

Sarcocystis Infection

Sarcocystis cruzi, *Sarcocystis hirsuta/Sarcocystis bovifelis*, and *Sarcocystis hominis* are coccidian protozoal parasites that commonly infect cattle. Carnivores are the definitive hosts while herbivores are the intermediate hosts. Cattle are infected by ingesting sporulated oocysts in carnivore feces. Once ingested, sporozoites are released into the gut, where they penetrate the mucosa and develop in arteries as meronts. The parasites are released from vessels as merozoites that then infect capillaries throughout the body. After a second round of replication, merozoites infect mononuclear cells and enter muscle and neural tissues to encyst. Clinical disease is uncommon in adult cattle but is associated with hemorrhage and edema due to the effects of intravascular maturation [273]. Signs include fever, ptyalism, lymphadenopathy, anemia, and abortion, with experimental oral infection [274].

Abortion is rare and has been reported in the third trimester; fetal hypoxia due to dam anemia, premature parturition due to $PGF_2\alpha$ release, pyrexia, or direct infection of the fetus are suggested etiologies [13, 19]. Parasitic cysts may be present in the placenta or the fetus, but this is not typical. Gross lesions may resemble *N. caninum* infection or be inapparent [19, 27]. Microscopic lesions are typically plentiful, consisting of necrosis in soft tissues and tissue cysts [27].

Miscellaneous Parasites

Abortion in herds infected by *Trypanosoma vivax* has been reported, likely a result of anemia and overall ill-health in the cow [275]. *Babesia bovis* and *Babesia bigemina* are associated with abortions as a result of pyrexia and fetal infection. Infected aborted fetuses are anemic and icteric, with swollen icteric livers [276]. *Theileria orientalis*, a cause of benign/non-transforming theileriosis, is associated with severe anemia and abortion in Australia, New Zealand, and Japan [277].

Fungi

The incidence of mycotic abortion varies widely [278]. In some areas fungal abortion is the most common abortion diagnosis [279]. Losses are sporadic as these organisms are not contagious. The degree of environmental contamination may increase the risk of fetal infection [27, 280].

A variety of ubiquitous environmental fungi cause abortions in cattle worldwide. Prevalence of fungal types and commonality of fungal abortion varies geographically. *Aspergillus* spp., *Zygomycetes* (*Absidia*, *Mucor*, *Rhizopus*, *Mortierella*), *Candida* spp., and *Pseudallescheria boydii* are among the more frequently reported [19]. *Aspergillus fumigatus* and other *Aspergillus* species are reported as the most common causes of mycotic abortion, with *Zygomycetes* the second most common [278]. It is thought that primary maternal disease in the respiratory or gastrointestinal tract leads to hematogenous spread to the placentomes, as lesions are first seen there and are most severe. Clinical signs in the dam are typically not seen. However, fetal abortion with *Mortierella wolfii* is associated with a severe post-abortion pneumonia in the dam [27, 280].

Abortions occur in the last trimester and they generally do not exceed 10% incidence in a herd [280]. They may be seasonal in association with increased environmental exposure. In the northern hemisphere, the winter and spring are most often associated with abortion [280]. Retained membranes are common. Infection causes severe thickening of cotyledons with extension into the intercotyledonary areas, imparting a cracked or leathery appearance to tissues. Cotyledons may have entrapped shreds of maternal caruncular tissue (see Figure 56.3). Fetuses may have minimal autolysis.

Placentitis develops slowly, interfering with fetal nutrition and often leading to fetal death. However, normal live calves may be born in cases of mild or even severe placentitis [13]. Fungal infection then extends to the fetus by infection of amniotic fluid. Fetuses may have numerous irregular tan epidermal plaques, particularly on the face, back, and sides (see Figure 56.8) [19]. Infections due to septate fungi (*Aspergillus* spp.) often cause raised, dry, and wrinkled skin lesions, while infections due to nonseptate fungi *(Mucor* spp., *Rhizopus* spp.) may cause flat, moist, white/red foci [20]. Because of the chronicity and severity of infection in placentomes, incarceration of cotyledons may prevent membrane detachment for up to 10 days. At this stage, complete placentomal necrosis may ensue, with retention of necrotic tissues, maceration, and/or pyometra [13].

Gross lesions are distinctive (see Figure 56.39). Placental lesions may mimic *B. abortus* or *Campylobacter* sp. infection [280]. Organisms may be cultured from the placenta, abomasal fluid, or lung. Placentas or, less commonly, abomasal fluids may have incidental fungal contamination. Diagnosis requires compatible lesions in addition to the presence of fungi.



Figure 56.39 Placenta with fungal placentitis and *Aspergillus* sp. Placentomes are swollen with abundant fibrinous exudate. *Source:* Courtesy University of Illinois Veterinary Diagnostic Laboratory.

Definitions

Asterisk denotes definitions from an authoritative source [52].

Abortion Expulsion of a conceptus incapable of independent life.* Calves born after day 260 are generally thought to be able to survive independently. Expulsion of the entire conceptus (complete abortion) or portions (incomplete abortion) may occur.

Allantois A secondary outgrowth of extraembryonic splanchnopleure from the ventral hindgut. The mesoderm is vascular.*

Amnion Extraembryonic somatopleure that is inverted around the embryo such that the ectoderm lines a cavity filled with amniotic liquor. The mesoderm is avascular.*

Birth Expulsion of a live fetus.

Blastocyst The stage in development after the morula, where the cells form a hollow sphere with an inner cell mass.*

BVDV Bovine viral diarrhea virus (bovine pestivirus).

Calf A live-born bovine.

Caruncle (plural carunculae) Discrete, monomorphic, regularly placed fleshy elevations of the inner uterine wall.

Chorion Extraembryonic somatopleure that is composed of an outer ectodermal trophoblast and is not part of the amnion. The mesoderm is avascular.*

Conceptus An inclusive term denoting the embryo or fetus and membranes.

Congenital Present at birth.*

Cotyledon Discrete, monomorphic, regularly placed elevations of the chorioallantois that are directly apposed and adhered to the uterine caruncles.

Embryo An animal in early development that does not have a form recognizable as a particular species.

Embryonic death Death of the conceptus prior to day 42.

Endemic abortion Abortion in a continuous pattern, at a rate greater than 5% throughout the year.

Epizootic/epidemic abortion A storm of abortions where greater than 10% of at-risk cattle abort in a short period of time, usually within two to three months.

Fetal death Death of a fetus prior to complete expulsion from the dam, subdivided into antepartum and intrapartum.*

Fetal period The stage of development between days 42 and 260, divided into early (days 42–120), middle (days 120–180), and late (days 180–260).*

Fetus An animal in development that has identifiable features of a given species.

Gestation The period of pregnancy; breeds typically vary in length, from 278 to 293 days.

Hamartoma A focally excessive overgrowth of mature normal cells in an organ that typically contains those cells.

IBR Infectious bovine rhinotracheitis virus (bovine herpesvirus 1).

Inner cell mass The group of cells in the blastocyst at one pole that develop into the embryo.*

Morula The early stage when eight or more cells are arranged in a solid mass without organization.*

Periparturient (peripartum) With reference to the dam, the time span occurring during the last 10% (30 days) of gestation to the first few weeks of calf life.

Placenta The fetal and maternal specialized organs of transfer between mother and offspring. The fetal placenta is the chorioallantois, while the general term "fetal membranes" includes the amnion as well.

Placentome The union of chorionic villi (cotyledon) and caruncle.

Premature parturition Delivery of an immature viable calf prior to completion of normal gestation (about 260 days).

Somatopleure The lateral and ventral body wall of the embryo composed of an outer ectoderm and an underlying mesoderm.

Splanchnopleure The embryonic layer formed by the fusion of the visceral mesoderm to the endoderm (definitive yolk sac wall).

Sporadic abortion Individual or small numbers of abortions occur at irregular intervals and typically do not exceed 3–5% annually.

Stillbirth Delivery of a dead fetus within the time of expected viability (after day 260).

Teratogen Anything, infectious or otherwise, that causes abnormal development in a conceptus.

Trophoblast The outer cellular layer composed of extraembryonic ectoderm through which all physiological exchange takes place.*

Zona pellucida The non-cellular layer surrounding the ovum.

Zygote A diploid cell formed from fusion of male and female pronuclei lasting until the first cleavage is complete.

Acknowledgments

Dr. F. Austin contributed to portions of this chapter regarding microbiology sampling of specific pathogens. The author wishes to thank Drs J. Cooley and J. Edwards for their figure contributions and editorial comments.

56.A Appendix

Abortion Diagnosis

Owner

Address

Phone/Fax/Email:

Veterinarian Name/Phone/Address:

Aborted calf: date aborted:_____Estimated due date: _____ Crown-rump length _____cm

Assistance needed for delivery? Y/N Vet involved? Y/N Calf alive when problem first noticed? Y/N

Gross fetal lesions:

Gross placental lesions:

Formalin fixed tissues: 10 parts fixative for each part tissue:	Fresh/chilled tissues: DO NOT FREEZE	Chilled samples: not in syringes
Brain (whole) □	Placenta (3 cotyledons) □ **bag separately**	Abomasum contents (3 ml)-
Liver □ Ileum □	The following **can be pooled if necessary**:	sterile tube □
Heart ventricle □ Adrenal □	Kidney (whole organ) □	Thoracic/abdomen fluid/heart blood (3 ml)-sterile tube □
Thymus (w/ trachea & esophagus) □	Liver (2"x2") □	Maternal serum, red top tube;
Lung (2 sections) □ Eyelid □	Lung (2"x2") □	at abortion □ 10–14 days later □
Placenta (3–4 cotyledons) □ Thyroid □	Spleen (1/2 organ) □	Herdmate serum, red top tube □
Lymph Node (mesenteric) □ Kidney □	Thymus (1 cm slice) □	(several cows are preferable)
Spleen □ Colon □	Adrenal (whole) □	10–14 days later □
Sk.muscle (limb,tongue & diaphragm) □		
T.foetus: TF pouch culture fetal fluids keep at rec. temp.		Fetal liver, kidney (frozen); bagged separately □

Cow/Dam

Breed:	Age/Tag#:	Lactation#:	Source/dam/sire:
AI ○ Bull ○ Hired bulls Y/N	Conception Date/Last Service:	Date of last normal parturition:	

Figure 56.A1 Abortion case submission form: abortion diagnosis.

Vaccines/ date given/ brand/ lot #/booster intervals
IBR: _____
BVD:_____
Neospora: _____
Lepto 5way: _____
Lepto hardjobovis:_____
Tritrichomonas:_____
C.fetus:_____
Chlamydophila:_____

Previous Abortions: Y / N When?
Workup Done? Y/N Results?

(DairyFarm)
Avg. days to 1st service:_____

Calving interval _____

Breedings per conception:

_____

Previous clinical disease in cow: Y/ N (circle all that apply): Present in last

Week	Month
3 mo.	6 mo.
12 mo.	24 mo.

Dam disease signs (circle all that apply): GI Respiratory Fever Mastitis

Retained placenta Anemia, dyspnea, emaciation (Anaplasmosis)

Changes in cow management/ pasture/ feeding regime:

Housing:

Forage: Grass hay _____ Legume Hay _____ Pasture _____Corn Silage _____ Other _____

Forage quality:

Concentrates/Minerals/Commodities/Selenium:

Any testing of feed/water for nutrients/toxins?

Feeding Program: Prepared or made on-farm Water source

adults in herd/ body condition:

#immature in herd/ body condition:

purchased in last: 6 mo._____ 12 mo. _____

Source:

Health problems:

Number of herd abortions in last:
Week_____ Month_____ 6mo._____
Associated with cold/hot weather?

Affects which trimester: 1st ○ 2nd○ 3rd○

Affects which cows: old young mixed

Problems with term calves:
Weak Ataxic Stillborn Malformed

Describe/how many:

Farm Abortion Frequency:
Sporadic ○
Recurrent ○
Recent problem ○

Pregnancy exams routinely done: Y / N

Postpartum exams routinely done: Y / N

_____ days after calving

Herd reproductive problems? Y / N What %?:_____ Circle all that apply: Anestrus Metritis

Repeat Breedings Irregular Cycles Retained Placentas Cystic Ovaries

Length of Breeding/calving season(beef):

Access to other cattle/ animals/ predators? Toxic plants on pasture?

Figure 56.A1 (Continued)

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Infectious Agents: Campylobacter

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Introduction

Campylobacter fetus subsp. *venerealis* is the etiologic agent of bovine venereal campylobacteriosis and is an important cause of infertility, death of the late embryo or early fetus, and sporadic abortion in cattle. This organism was first recognized in the early 1900s as a cause of abortion in cattle and was initially called *Vibrio fetus* [1]. Based on differences in clinical presentation the agent was further classified as *Vibrio fetus* subsp. *intestinalis* and *Vibrio fetus* subsp. *venerealis*. *Vibrio fetus* subsp. *intestinalis* was associated with sporadic abortions due to bacteremia from the intestinal tract of the pregnant cow. *Vibrio fetus* subsp. *venerealis* was also associated with occasional abortions, but the primary importance of infection was a significant decrease in herd fertility. However, in the 1960s there was evidence that many of the *Vibrio* species were sufficiently different to warrant classification under a separate genus, *Campylobacter* [2]. Consequently, *Vibrio fetus* subsp. *intestinalis* was renamed *Campylobacter fetus* subsp. *fetus* and *Vibrio fetus* subsp. *venerealis* was renamed *Campylobacter fetus* subsp. *venerealis*. "Vibrio" is still a commonly used term to refer to the venereal disease caused by *Campylobacter fetus* subsp. *venerealis*.

Etiology

C. fetus subsp. *venerealis*, a slender, motile, Gram-negative, comma-shaped or S-shaped rod that appears as "seagull wings" in silhouette, requires a microerophilic environment for growth [2, 3]. *Campylobacter* spp. require an atmosphere of 10–20% CO_2 with reduced oxygen concentration at 5% or less and optimal growth occurring at 37 °C [2]. There are two biotypes within the subspecies, biotype *venerealis* and biotype *intermedius*. These biotypes are differentiated by the production of hydrogen sulfide with lead acetate strips when organisms are grown in media containing cysteine. Biotype *intermedius* produces hydrogen sulfide, whereas biotype *venerealis* does not. Both biotypes are capable of causing disease; however, biotype *intermedius* appears to be less pathogenic than biotype *venerealis* [4].

Pathogenesis

C. fetus subsp. *venerealis* is primarily transmitted by the venereal route. Bulls serve as carriers and are the major mode of transmission of the organism through a naive herd. The rate of transmission from infected bulls to naive cows is quite high, approaching 100%. The bacterium may be spread to cows and heifers at the time of artificial insemination (AI) with infected semen. Direct transmission from cow to cow is unlikely, although contaminated insemination equipment may act as a fomite, allowing for transmission of the organism [5]. Campylobacteriosis may be spread from bull to bull if contaminated semen collection equipment is not sufficiently cleaned between bulls [4]. In addition, transmission has been reported to occur from bull to bull due to contaminated bedding [6].

Bull

The role of bull age in the course and duration of disease is unknown. One study demonstrated no difference in duration of infection between bulls 5.5–6 and 3.5–4 years

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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of age [7]. However, another study revealed a higher incidence of infection in bulls greater than 6 years of age [8]. It has been speculated that younger bulls may be more resistant to infection and more likely to clear infection, and clear infections within a few weeks. In addition, it has been speculated that older bulls are more likely to become persistent carriers due to an increase in the number and depth of crypts in the preputial epithelium [4]. However, more recent work evaluating the surface architectural anatomy of bulls revealed no differences in penile and preputial epithelial anatomy between young and old bulls [9].

The prepuce of bulls serves as the natural reservoir of infection to naive cows through natural mating [10]. The addition of carrier bulls poses a significant risk to a naive herd, although the disease is most commonly seen in newly introduced cows and heifers in herds where bovine campylobacteriosis is endemic [11].

Cow/Fetus

Venereal campylobacteriosis causes low reproductive efficiency, resulting in severe economic loss to a cattle operation [12]. This is associated with temporary infertility with mild endometritis, death of the early embryo or early fetus (30–70 days of gestation), and sporadic abortions [13, 14]. After exposure the bacteria colonize in the mucosa of the anterior vagina and cervix of cows [10]. The infection then spreads to the uterus and oviducts under the influence of progesterone. Fertilization and development of the embryo are not directly affected by *C. fetus* subsp. *venerealis*. The resulting inflammatory response in the uterus and/or oviducts results in early embryonic death. The majority of pregnancy loss occurs between 30 and 70 days of gestation [13, 15]. Reactions associated with heat-stabile endotoxins produced by *C. fetus* subsp. *venerealis* are thought to be responsible for the occasional mid-term abortion [4]. *C. fetus* subsp. *venerealis* has the ability to reach the placenta and the amniotic fluid and cause severe placentitis and fetal hypoxia [16]. Cows and heifers are normally capable of mounting an immune response, clearing the infection, and returning to normal fertility four to eight months after the initial infection [13].

Immune Response

Infection with *C. fetus* subsp. *venerealis* in cows and bulls does not produce significant systemic antibodies during or after infections [17]. Unlike bulls, cows and heifers infected with *C. fetus* subsp. *venerealis* produce a local antibody response in the vagina and uterus. Heifers challenged with *C. fetus* subsp. *venerealis* had transient agglutinating and immobilizing immunoglobulin (Ig)M antibodies followed by persistent IgA and IgG antibodies in vaginal secretions and predominantly IgG antibodies in uterine secretions [17, 18]. Both IgA and IgG antibodies to *C. fetus* subsp. *venerealis* surface antigens immobilize and mediate neutrophil and mononuclear cell killing of the bacteria [19]. The production of IgG in the uterus directs clearance through opsonization and phagocytosis. In the lower reproductive tract, the IgA produced helps to immobilize the bacteria and block adherence of the organism. Conversely, IgA may also be responsible for blocking opsonization and promoting persistent colonization of the lower genital tract. Because of the stronger antibody response in cows and heifers, prolonged infections in females are not as persistent as they are in bulls [18, 20]. However, in the rare case of the chronic carrier cow, the bacterium may be able to elude the immune response and be maintained in the cervix through a full gestation, although this is quite unusual (<1% of infected females) [6]. Persistence of infection in the cervix and vagina in the presence of an immune response may be partly due to antigenic changes in the organism that allow for avoidance of the host immune response. Antigenic changes in the S-layer, a regular array of high-molecular mass surface proteins that compose the outermost part of the cell envelope, result in the expression of different immunodominant epitopes and persistence of the organism [4].

Clinical Signs

Herd

A herd history of poor conception rates, infertility, or second trimester abortions is commonly one of the first indications that bovine venereal campylobacteriosis could be a problem. Trichomoniasis, poor detection of estrus, and bull subfertility or infertility should also be considered as possible differential diagnoses. A confirmatory diagnosis of bovine venereal campylobacteriosis should be attempted through laboratory evaluation of samples obtained from the herd. Samples for diagnosis include tissues from aborted fetuses, placenta, vaginal mucus samples, vaginal washings, and preputial scrapings.

When beginning a diagnostic evaluation of a herd, it seems advantageous to begin with the breeding males in the herd due to the fewer number of males and the self-limiting type of infection associated with females. Thus preputial scrapings should be obtained from all herd bulls. If bulls are not available, vaginal mucus samples should be obtained from a representative sample of cows and heifers and submitted to the diagnostic laboratory.

Cow

In females the main clinical signs are related to infertility. Venereal campylobacteriosis is characterized by repeat breeding, irregular estrous cycles, prolonged calving seasons, and reduced calf crops [13, 21]. The average number of services per conception is extended to 2.5–3.5. When infection is introduced into a naive herd, the initial pregnancy rates are quite low. As the breeding progresses, the conception rate of the herd improves as females clear the infection. However, the resulting calving season is extended as more cows conceive later in the season. Infected cows show few clinical signs of infection. Most infected cows do not exhibit vaginal discharge; however, a mucopurulent secretion may be detected in the occasional cow [4]. As previously stated, the majority of pregnancy loss occurs between 30 and 70 days of gestation; the uncommon midterm abortion, four to eight months of gestation, due to *C. fetus* subsp. *venerealis* has also been reported [13, 15]. Abortions appear to be more commonly associated with biotype *intermedius* than with biotype *venerealis*. When midterm abortions due to *C. fetus* subsp. *venerealis* are reported, there is typically a recent history of infertility in the herd [4].

Bull

Infection with *C. fetus* subsp. *venerealis* in bulls is not apparent as bulls do not exhibit clinical signs of infection. Although genital infections in mature bulls may persist for months or years, there are no alterations in semen quality or production [22–24].

Pathology

The pathology associated with infection of *C. fetus* subsp. *venerealis* is limited to the reproductive tract. Endometritis, mild cervicitis, and salpingitis are usually the only lesions seen in cows and heifers [25]. Grossly the cervix may appear reddened and the uterus may produce a small amount of mucopurulent exudate that can extend through the cervix into the vagina. Histologically, the lesions have mild infiltration of inflammatory cells (plasma cells and foci of lymphocytes in the stroma) with slight desquamation of the superficial epithelium with no significant vascular changes [25]. Involvement of the endometrial glands is minimal, although cystic glands with slight periglandular fibrosis have been noted on clearance of infection [25].

No gross abnormalities are observed on the penis and prepuce of infected bulls. Histologically, there is only a diffuse infiltration of mononuclear cells within the lamina propria of the penile and preputial epithelium [22]. Plasma cells are located in clusters at the apex of the dermal papillae [7]. However, the infiltration of subepithelial lymphocytes and plasma cells has been found in both infected and uninfected bulls [22, 24, 26].

The most common fetal lesions associated with *C. fetus* subsp. *venerealis* are neutrophilic bronchopneumonia and interstitial pneumonia [16]. Other commonly observed lesions include non-suppurative interstitial enteritis, hepatitis, pericarditis, myositis, myocarditis, serositis, occasional abomasitis, and meningitis [16, 27, 28].

Diagnosis

It is important to be able to differentiate *C. fetus* subsp. *venerealis* from *C. fetus* subsp. *fetus*. The natural habitat for *C. fetus* subsp. *fetus* is the intestinal tract of cattle, but it can also cause sporadic abortions [29]. Differences in the epidemiology and clinical relevance of these bacteria make accurate detection imperative. The traditional means for differentiating between both species, and the recommended international standard, is based on tolerance to 1% glycine [30]; *C. fetus* subsp. *venerealis* is sensitive to glycine whereas *C. fetus* subsp. *fetus* is tolerant to glycine. However, the glycine tolerance characteristic may not be completely reliable for differentiation of the two subspecies as it has been demonstrated that glycine tolerance can be acquired by transduction or mutation [31]. In addition, glycine-tolerant variants of *C. fetus* subsp. *venerealis* have been described and designated as *C. fetus* subsp. *venerealis* biotype *intermedius* [30, 32]. Glycine-sensitive *C. fetus* subsp. *fetus* isolates have been detected as well [33]. Another method of differentiation between the two organisms is a hydrogen sulfide production test, as *C. fetus* subsp. *fetus* produces hydrogen sulfide and *C. fetus* subsp. *venerealis* does not [34].

Sample Collection

Bull

A simple method for collecting preputial smegma is to take a preputial scraping. The most common method for collecting a preputial scraping involves using a plastic AI pipette, which is fast, easy, and inexpensive [14]. The tip of the AI pipette is placed into the proximal preputial fornix. Scraping of the prepuce should be somewhat vigorous in an effort to loosen epithelial cells from the penile and preputial surfaces and include 10–15 strokes. A 12-ml syringe should be attached to the distal end of the AI pipette, and the clinician should apply negative pressure to the pipette throughout the scraping procedure [14]. A small amount

of hemorrhage may be seen following preputial scraping but usually does not indicate any harm to the bull. As the pipette is being withdrawn from the preputial cavity, the negative pressure on the pipette should be released gently. An adequate sample consists of a 2- to 5-cm column of cloudy smegma within the pipette. A plastic sheath may be used to minimize contamination prior to introducing the pipette into the preputial cavity. The Pizzle Stick (Lane Manufacturing) provides an alternative collection tool to the traditional AI pipette. This tool is used in the same manner as the AI pipette; however, the corrugated grooves located on the tip of the stick allow for the collection of smegma. Historically, other methods of collecting smegma from the preputial cavity of bulls include preputial lavage (douching) and swabbing the penile and preputial epithelium with the penis held in extension.

Cow

A cranial vaginal mucus sample can be obtained from the cow by using an AI pipette with a plastic sheath to minimize contamination. A 12-ml syringe is placed on the distal end of the AI pipette, which is then placed into the cranial vaginal fornix of the cow. Negative pressure is placed on the syringe while the pipette is gently moved within the vaginal fornix. Collection of vaginal mucus samples should be performed around the time of estrus. Culturing of vaginal mucus samples is considered most useful diagnostically when samples are obtained early in the course of infection before immunity develops [4].

Fetus

Samples that should be collected from an aborted fetus include abomasal fluid, lung, liver, heart, intestine, and placenta. Abomasal fluid from aborted fetuses serves as the best diagnostic sample. Fetal membranes may also be useful, but diagnosis is often complicated due to the presence of contaminating bacteria [4].

Sample Transport

Proper handling of preputial scrapings, vaginal mucus samples, or samples from aborted fetuses and fetal membranes is critical for accurate diagnosis of *C. fetus* subsp. *venerealis*. In order to optimize recovery of the organism, it is best to have samples at the laboratory within six hours of sample collection. *Campylobacter* spp. require a complex support medium, antimicrobials to suppress growth of commensals and contaminants, and near-anerobic conditions [35]. The success of culture of *Campylobacter* spp. is directly related to the choice and availability of media. Several different transport enrichment media (TEM) are available for isolation and culture of samples for *C. fetus* subsp. *venerealis*. These TEM include Weybridge medium, Cary–Blair medium, Clark's selective medium, Australian TEM, Blaser–Wang medium, Lander's transport medium, Campy-Bac, and Thomann transport and enrichment medium [3, 14, 36, 37]. It is best for clinicians to contact their local diagnostic laboratory for preferred media and sample handling prior to collection and submission of samples. In many cases the laboratory will supply the transport media and instructions for its use. Once in the TEM, the sample is safe, to some degree, from small changes in temperatures. If an inoculated TEM is to be in transit more than four hours, it is recommended that the sample not be incubated above room temperature until it reaches the diagnostic laboratory [14, 38].

Samples can be pooled to decrease the cost of testing animals when permitted by the diagnostic laboratory. Sensitivity of the quantitative polymerase chain reaction (qPCR) has been shown to be comparable for the pooling of up to five samples with that of a single unpooled sample [39]. However, care should be taken to contact the local diagnostic laboratory before pooling samples, as not all allow pooled samples or, if they do, many prefer to pool the sample themselves.

Temperature during transport may be more critical depending on the time it takes for the sample to arrive at the diagnostic laboratory. It has been shown that sensitivity of qPCR is highest for short-term transport (2–48 hours) when shipped at either 4° C or 30° C, but long-term transport (96 hours) has the highest sensitivity at −20° C [39].

Diagnostic Techniques

Antigen or Antibody Detection

Antigen detection techniques are most often used to identify an active carrier of venereal campylobacteriosis in bulls, although antibody detection may be more useful for detection of the organism in cows. The detection of antigen includes the fluorescent antibody test, which is most commonly used for preputial scrapings from bulls [36]. The sensitivity and specificity are reported to be 93 and 88.9%, respectively [40]. An immunohistochemical technique has also been developed, with a sensitivity of 94% for detecting *C. fetus* in aborted fetal tissues [41].

Systemic antibody responses are not helpful because they are often due to non-pathogenic *Campylobacter* spp. One specific antibody detection method is the vaginal mucus agglutination test to detect antibodies in preputial

scrapings, vaginal mucus, and aborted fetal fluids [42]. However, due to the low sensitivity of the test, it is best used for herd evaluation utilizing vaginal mucus samples collected between 37 and 70 days after infection [3]. In addition, the specificity of this test may be negatively affected by the presence of blood or an unrelated inflammation [4]. A vaginal mucus sample that causes complete agglutination at a dilution of 1 : 25 is considered a positive diagnosis [4].

Specific IgG antibodies have been detected via enzyme-linked immunosorbent assay (ELISA) in order to measure the humoral immune response in cattle following vaccination against *C. fetus* [43, 44]. An ELISA has also been used to detect IgA antibodies in vaginal mucus from cows following abortion [45], and a monoclonal antibody-based ELISA has been developed as a screening test for detecting *C. fetus* subsp. *venerealis* in preputial scrapings and vaginal mucus samples [46, 47].

Isolation in Culture

Campylobacter spp. are not easily cultured and isolated due to the organism's fastidious nature and special requirements for growth in media [3, 48]. Once at the laboratory the TEM is streaked onto selective media and incubated in a microerophilic atmosphere (6% O_2, 7% CO_2, 7% H_2, 80% N_2) at 37 °C. The media plates should be incubated for at least six days before being called negative. In many cases, colonies of *C. fetus* subsp. *venerealis* will be visible within three days of inoculation. Colonies are round, raised, and 1–3 mm in diameter. These colonies may initially appear clear but become opaque on further incubation. Phenotypic characteristics are most commonly used for identification. *C. fetus* subsp. *venerealis* isolates are oxidase positive, catalase positive, resistant to nalidixic acid but sensitive to cephalothin, and grow at 25 °C but not at 42 °C. It is imperative to differentiate between *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* due to potential sample contamination. These two subspecies of *C. fetus* only differ in that *C. fetus* subsp. *venerealis* does not grow in the presence of 1% glycine whereas *C. fetus* subsp. *fetus* does grow in 1% glycine [4]. As previously mentioned, the glycine tolerance characteristic may not be completely reliable for differentiation of the two subspecies as it has been demonstrated that glycine tolerance can be acquired by transduction or mutation [31]. A passive filtration technique during culture may increase the recovery rate of *C. fetus* subsp. *venerealis* while decreasing the contamination from other organisms. This is accomplished by applying a mixed cellulose ester membrane filter over the top of a blood agar plate and inoculating with the sample. The plate is incubated right side up at 37° C for 30 minutes, allowing for motile organisms to cross the filter into the media. The filter is then discarded and the plate returned to the same environment for an additional 72 hours of culture [49].

Direct examination via darkfield microscopy and Gram's staining of abomasal contents from aborted fetuses may be a useful additional tool for diagnosing abortions due to *C. fetus* subsp. *venerealis*. Darkfield microscopy may allow observation of the morphologic traits and darting motility of *C. fetus*. Gram staining reveals a Gram-negative, comma-shaped or S-shaped rod that appears as "seagull wings" [2, 3]. *Campylobacter* spp. are more easily visualized by Gram's stain when counterstained with carbolfuchsin rather than safranin, the standard counterstain [2–4]. Darkfield microscopy and Gram staining may not be as beneficial for other samples.

PCR

Molecular methods for differentiating between *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* have been developed to validate the results of phenotypic differentiation in order to avoid misidentification [29]. Most of these polymerase chain reaction (PCR) assays use the colonies grown from the cultured vaginal mucus or preputial scrapings [14]. However, Thomann transfer and enrichment medium was developed for the enrichment of *C. fetus* subsp. *venerealis* and to allow direct PCR amplification from media if overgrowth with contaminants occurs [37]. Differentiation of *C. fetus* spp. is achieved by a specific PCR assay [50]. This assay utilizes a species-specific 764-bp amplicon that is produced with primers MG3F and MG4R with the DNA of both species of *C. fetus*. Subsequently, primers VenSF and VenSR are used for differentiation. The identification of *C. fetus* subsp. *venerealis* is based on the presence of a 142-bp amplicon that is not formed by *C. fetus* subsp. *fetus* [50]. However, the reliability of this PCR assay has been called into question [30] due to misidentification in up to 10% of strains [51]. Therefore this PCR assay may best be used to confirm traditional phenotypic characterization of *C. fetus*.

Other studies have focused on the use of a multiplex PCR for differentiation of *C. fetus* spp. [51]. Multiplex PCR consists of multiple primer sets within a single PCR mixture. These primer sets produce amplicons of varying sizes that are specific to different DNA sequences, which are observed as distinct bands when visualized by gel electrophoresis. However, utilization of multiplex PCR will require further validation with regard to a larger pool of clonal strains [52, 53].

Cloning and nucleotide sequencing, with and without PCR, are becoming more useful methods for genotyping bacterial isolates [54]. In fact a novel 5′ *Taq* nuclease PCR assay using a 3′ minor groove binder DNA probe has been developed for the direct detection of *C. fetus* subsp. *venerealis* in clinical samples from cattle [55]. Molecular methods to

discriminate between the two subspecies of *C. fetus* have also utilized the discovery of an 80-kb genomic sequence (consisting of type IV secretion system [T4SS] components, putative plasmid genes, and other proteins) and an insertion element (ISCfe1) unique to *C. fetus* subsp. *venerealis* [56, 57].

Prevention, Control, and Treatment

Prevention

Vaccination programs are the most effective means of preventing campylobacteriosis in a cattle herd and should include vaccination of bulls as well as cows and heifers [13]. Initial immunization of females requires two injections of bacterin, with the second injection coming within three weeks of breeding. Annual boosters should also be administered within three weeks prior to breeding [14]. This prebreeding schedule of vaccination increases antibody levels (IgG_1 and IgG_2) at the time when exposure is most likely to occur and helps protect against uterine infection [14].

Many different *C. fetus* vaccines are available for use in cattle, including both oil-adjuvanted and aluminum hydroxide-absorbed vaccines [13]. Bacterins in oil adjuvant are more effective and provide a longer duration of immunity and protection after a single dose [58]. Two commercial aluminum hydroxide-absorbed vaccines were evaluated in herds in Argentina and were found to be ineffective at preventing reproductive loss due to *C. fetus* [13, 59]. Subcutaneous administration of a single dose of an oil-adjuvanted *Campylobacter* vaccine demonstrated protection in heifers from reproductive losses. However, oil-adjuvanted vaccines cause localized granulomas and granulomas at the injection site [13].

Vibrin™ (Zoetis) is the only oil-adjuvanted *C. fetus* bacterin available in the USA that has been evaluated in bulls [13]. Two 5-ml doses, which is 2.5 times the dosage recommended for vaccination of cows, should be administered subcutaneously to breeding bulls at four-week intervals beginning eight weeks prior to the breeding season. Annual revaccination at the same dose is recommended four weeks prior to the breeding season [13, 60].

Control

Elimination of natural breeding and the use of AI is the best means of controlling bovine venereal campylobacteriosis. However, producers often find this a difficult recommendation to follow. If AI is used the semen should be adequately screened for *C. fetus* subsp. *venerealis* and have antibiotics added to the semen extender to ensure that contaminated semen does not allow reintroduction of the bacterium into the herd. If AI is not possible, the previous battery of bulls should be replaced with virgin bulls [14]. The entire bull battery should be tested at the end of the breeding season, with all positive bulls being culled.

Treatment

Because of the lack of availability and effectiveness of certain antibiotics (streptomycin), the treatment of animals infected with *C. fetus* subsp. *venerealis* has focused on immunotherapy to clear the infection, which involves immunizing animals with an appropriately adjuvanted antigen. Systemic immunization of cows that have been actively or passively immunized with *C. fetus* subsp. *venerealis* has cured these animals of the vaginal carrier state [24, 61, 62]. In addition, systemic immunization may cure, or clear infection for nine weeks or more, in some bulls infected with *C. fetus* subsp. *venerealis* [23, 63, 64]. Immunization induces both local and systemic IgG antibody production, which prevents microbial adherence, immobilizes the bacteria, activates complement, and opsonizes bacteria for phagocytosis [24]. The oil-adjuvanted vaccine preparations are most often used for therapeutic immunotherapy.

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58

Infectious Agents: Tritrichomonas

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Introduction

Trichomoniasis is a venereal disease in cattle. The causative agent is an extracellular protozoal agent, *Tritrichomonas foetus*. Infections occur in female and male cattle. However, the bull is the asymptomatic carrier, while the cow experiences clinical signs manifested as embryonic death, abortion, metritis, pyometra, and transient infertility. Older bulls are often unable to clear the infection and become chronically infected carriers [1]. A routine breeding soundness examination does not include *T. foetus* testing, so a *T. foetus* infected bull may pass such an examination. Both "trichomoniasis" and "trichomonosis" are commonly used to describe *T. foetus* infections in cattle [2]. Additionally, *T. foetus* is able to survive the freezing process used when freezing semen. Therefore only bulls that are tested and determined to be negative for *T. foetus* are utilized in certified bull studs. Using frozen semen from *T. foetus* negative bulls is considered a method of control. During the last century, the reported number of cases of *T. foetus* has increased dramatically. This increase is most likely associated with increased movement and demand, drought, and the development of improved modalities of identification of *T. foetus* infected animals. Swift identification and eradication of positive animals is the current control modality. With the identification and removal of infected animals being key to control of this disease, many states have implemented rules and regulations regarding the testing and movement of *T. foetus* positive animals. The economic losses seen with herd outbreaks of *T. foetus* can be devastating. In the USA there is currently no approved treatment for trichomoniasis in cattle; however, new therapy methods are currently being investigated.

Organism

The identification of *T. foetus* is credited to an Italian scientist Mazzanti in 1900, when he identified the organism in two cows and one heifer that were slaughtered due to infertility [3]. At the approximate time of the discovery of *T. foetus*, the disease brucellosis emerged and most likely became the predominant focus of research at the time [3]. In the 1920s work with *T. foetus* reemerged in Germany. In 1928, Riedmüller while studying aborted fetuses identified trichomonads in several of the affected fetuses. Following this study the name *Tritrichomonas foetus* was coined [3]. The first identified case of trichomoniasis in the USA was reported in Pennsylvania in 1932 from vaginal smears of several cows experiencing reproductive failure [3]. The organism has been identified in six of the seven continents, the exception being Antarctica, and is considered endemic in the USA west of the Mississippi River [2, 4].

T. foetus is an extracellular protozoan and an obligate parasite of the reproductive tract of cattle. *T. foetus* belongs to Trichomonadea, which comprises a class of amitochondriate protists belonging to the phylum Parabasalia [5]. *T. foetus* is a small flagellate whose size ranges from 10 to 25 μm long and 3–15 μm wide with a single well-developed laterally located undulating membrane spanning the length of the entire organism [6, 7]. Additionally, *T. foetus* has three less well-developed anteriorly located flagella and one posteriorly located flagella [6, 7]. Also, *T. foetus* undergoes two lifestyle stages, the pseudocyst and the trophozoite. The pseudocyst usually forms in times of unfavorable environmental conditions, such as following a decrease in nutrients, administration of drugs, or abrupt changes in temperature [5]. The trophozoite internalizes the flagella and the organism rounds up forming pseudocysts with no cyst wall surrounding the

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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cell. These *T. foetus* pseudocysts are able to adhere to host cells and the pseudocyst adhesion rate is reported to be even higher than for the trophozoite form [8]. Reversibility assays have found that the pseudocysts can revert to the trophozoite form when conditions are favorable [5].

T. foetus is reported to infect other species such as domestic cats and colonize the colon, resulting in chronic irritation and diarrhea [9]. Additionally, *T. foetus* and *T. suis* are described as inhabitants of the porcine gastrointestinal system and the nasal mucosa [5]. It is believed that *T. foetus and T. suis* are synonymous [10]. Care must be used when identifying trichomonads microscopically as fecal trichomonads are extremely similar in size, shape, and characteristic movement and may even be the same. These similarities decrease the specificity of culture to accurately identify *T. foetus*, and so testing utilizing polymerase chain reaction (PCR) is necessary to truly differentiate different trichomonads. Prevention of disease within a herd is best done by institution of biosecurity protocols including testing of all non-virgin bulls and replacements and removal of infected animals, establishment of a closed herd, utilization of artificial insemination, and vaccination.

Transmission and Clinical Signs

As with the majority of sexually transmitted diseases, the male is the asymptomatic carrier, while the female experiences the reproductive losses. Transmission can occur via infected male to female or infected female to male during coitus. Transmission from infected female to infected female via uninfected bulls is possible if breeding occurs within the narrow time frame of 20 minutes [11, 12]. Infection of the female by an infected male requires only one breeding; however, the infection of an uninfected male by an infected female can require up to six breedings [4]. Infection via the use of contaminated artificial insemination guns and vaginal speculums is rare but has been reported [13]. In the bull, *T. foetus* inhabits the epithelium of the prepuce and penis [11]. Additionally, *T. foetus* has been isolated in the distal urethra of the bull [11]. It was previously speculated that there is an increase in the depth of the epithelial folds within the prepuce of aging bulls, usually referred to as "crypts." The presence of increasing depth of the epithelial folds of the prepuce has since been disproven via a study by Strickland et al. [14], which found that there were no significant differences observed in the preputial histology of bulls less than two or greater than five years of age.

In the cow, *T. foetus* inhabits the cervical mucus and eventually spreads throughout the entire reproductive tract [11]. Infection occurs following breeding with an affected bull. Once *T. foetus* is present in the cervical mucus, the organism moves from the cervix and vagina to the uterus during estrus and within weeks the entire reproductive tract is colonized [15]. *T. foetus* can be identified in the tract of a female as soon as four days post infection, with no apparent negative impact on conception or maternal recognition of pregnancy associated with the presence of *T. foetus* [13]. The number of parasites present in the cervicovaginal mucus fluctuates depending on the stage of the estrous cycle. The numbers of trichomonads are highest several days before estrus is observed [16]. Inflammation associated with infection of *T. foetus* can result in vaginitis and cervicitis. Infection ultimately leads to reproductive failure in the female, with embryonic death and abortions occurring most often within 95 days of exposure [13, 17]. Less than 1% of infected cows become chronically infected [18, 19]. Chronically infected females are defined as females that have remained infected for up one year post-infection [19]. These chronic carriers can serve as a source of reinfection for bulls and other cows [16].

Infected bulls do not suffer from any penile or preputial lesions and there are no reported negative impacts on libido [11]. However, there is a negative impact on motility according to a study by Ribeiro et al. [20]. This study found that *T. foetus* released cytotoxic substance, resulting in a significant decrease ($P \leq 0.05$) in progressive motility [20]. A study conducted in Florida documented that the mean age of infected bulls was 5.5 years $\pm$ 1.6, with the mean age of uninfected bulls being approximately 1.5 years younger [13]. Routine bull breeding soundness examinations (BBSEs) cannot identify infected bulls via the spermiogram as there is no negative impact of the trichomonads on semen morphology and motility may be lower but considered adequate. Annual BBSEs are an ideal time for collection of preputial smegma for testing of *T. foetus*. If the producer maintains a closed herd, has experienced no reproductive losses or decreased calf crop, and a previous negative trichomoniasis test had been obtained, it is less vital but remains a good recommendation to retest bulls every year.

Infection with *T. foetus* can cause a variety of reproductive issues in the female such as embryonic death, transient infertility, metritis, pyometra, and abortions [21]. The majority of embryonic death occurs at approximately day 17, at which time maternal recognition of pregnancy has occurred [16]. Although abortions occurring after three months of gestation are uncommon, placental lesions have been reported [22]. A clinical sign often noticed by the producer is an increased interestrus interval, which ultimately leads to a prolonged breeding season and a stretched out calving season. As most of the reproductive loss is manifested as embryonic loss, very few fetal abortions will be visualized. If a controlled breeding season is not already implemented, then affected herds can go undetected for

several months and even an entire calving year. A prolonged calving season will then occur, with most calves born late in the season. Economic losses from embryonic loss and lower weaning weights due to decreased time for average daily gain can be devastating. The effect of the prolonged calving season and late calving dates can be felt years later as the same cows will continue to have calves late in the calving season. In herds where trichomoniasis has gone undetected for a period of time, the results may be less dramatic because of temporary herd immunity. In such cases, the calf crop may only be reduced 5–10%. However, this loss is still enough to have a substantial economic impact on feeder calf breakeven price and profit per brood cow [23, 24]. The economic impact attributed to direct, secondary, and indirect losses is approximately USD $650 million annually in the USA and $300 million in the state of Texas alone [12, 25, 26].



Figure 58.1 Infusion pipette and Pizzle Stick used for collection of smegma from bulls potentially infected with *T. foetus*.

Sample Collection, Testing, and Diagnosis

As there is no currently approved treatment for *T. foetus* in the USA, control is reliant on the prompt identification and removal of positive bulls, followed by the institution of strict biosecurity. Identification of the organism is performed via the collection of preputial smegma in bulls and cervicovaginal secretions in female cattle [27]. Identification of the organism is also possible in aborted placental and fetal abomasal fluids; therefore all abortions should be investigated [16]. The rationale for obtaining samples from whole herds of cows is not logical due to the limited window of presence of the trichomonad in the vaginocervical mucus after infection, which ranges from 12 to 20 hours post-infection [28]. The preputial smegma from the bull is the most readily accessible and most reliable sample to obtain.

Prior to collection of preputial smegma, the bull's preputial hairs should be examined and cleaned of any debris to decrease the risk of fecal contamination of the sample. There are currently several methods of preputial smegma collection, including the Pizzle Stick® (Lane Manufacturing, Denver, CO), mare infusion pipette with attached 10-ml syringe, TRICHIT™ (Morris Livestock Products, Delavan, WI), microscope slide, gauze collection, and preputial wash. For the sake of ease, the most utilized collection methods are those that do not require extension of the penis, such as but not limited to the mare artificial insemination pipette and the Pizzle Stick, as shown in Figure 58.1.

Regardless of the collection method utilized it is imperative to ensure an adequate sample is collected from the prepuce, level with the midshaft of the penis, extending all the way to the fornix of the prepuce. Proper collection requires equipment in which the bull can be safely restrained with adequate access to the penis and prepuce. A study performed by Parker et al. [29] showed that choice of the side of collection was more important than the number of scrapes along the prepuce and shaft. This study stated that the most adequate samples are obtained when the practitioner collects from the side from which their dominant hand is located; therefore a right-handed individual should attempt to collect the bull from the right side [29]. For best ease of passage of the collection device, the thumb of your non-dominant hand is placed at the ventral aspect of the preputial orifice. Once your thumb is in place it acts as a guide for the collection device for placement into the preputial cavity (Figure 58.2). The hand can be moved from the preputial orifice and the collection device gently guided to the preputial fornix. Once the device is positioned at the fornix of the prepuce, the non-dominant hand massages at the level of the fornix (Figure 58.3), while the dominant hand manipulates the collection device five to ten times to ensure an adequate sample is collected.

Samples should be immediately placed in a collection device containing Modified Diamond's media. Media containers include a two-chambered pouch (In Pouch®) and a trichomoniasis transport tube containing Modified Diamond's media (Figure 58.4).

The sample should be free of gross debris to help ensure no fecal contamination occurs. If it is grossly contaminated the sample should be discarded and a new sample collected. It is important not to leave the sample in direct sunlight or place in a cooler or refrigerator as hot and cold will kill the organism. The death of the organism is less of a problem since the development of PCR, which will identify



Figure 58.2 Positioning of smegma collection device in preputial orifice of a bull.



Figure 58.3 Positioning of smegma collection device at preputial fornix of a bull.



Figure 58.4 Smegma collected with the Pizzle Stick being transferred into Modified Diamond's media (*T. foetus* transit tube).

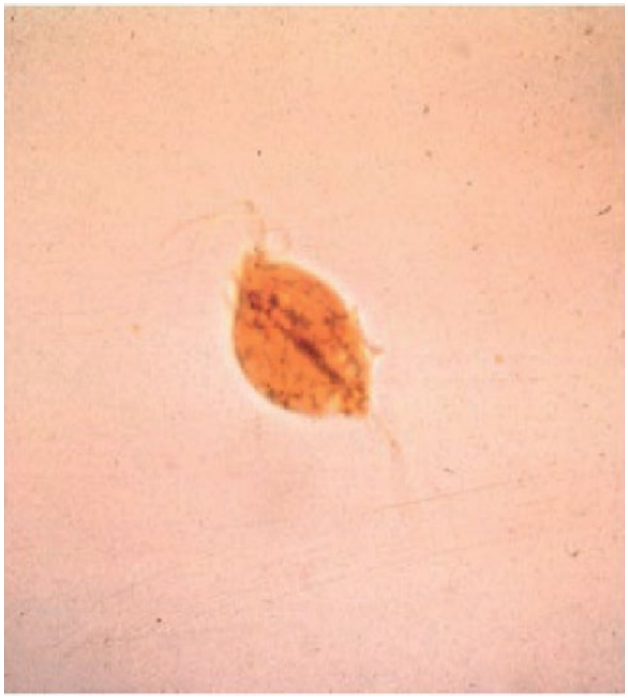


Figure 58.5 *T. foetus. Source:* Courtesy of Maarten Drost.

the organism's DNA. However the dead organisms will not be identifiable on culture. Testing accuracy depends on the relative sensitivity and specificity. Sensitivity is defined as the ability of the test to correctly identify the positive animals with a positive test result. Specificity is defined as the ability of a test to correctly identify negative animals as truly negative.

Diagnostic testing for identification of *T. foetus* includes culture in Modified Diamond's media with bright field microscopic identification and PCR [1]. Culture alone relies on the microscopic visualization of the organism's characteristic jerky, rolling progressive movement [16]. Direct visualization of the organism is possible (Figure 58.5); however, due to the variable number of organisms present, a 25% decrease in sensitivity is appreciated [16]. Overall sensitivity of culture is lower than PCR due to the possible contamination of fecal trichomonads as the size and shape are almost visually undistinguishable. The PCR alone possess a

high degree of sensitivity with the ability to identify as few as 50 trichomonads per milliliter of sample [6].

The gold standard for identification of the organism has been PCR following a 48-hour culture period. The combination of *in vitro* culture followed by PCR has increased the sensitivity of the testing modality [6]. However, new tests have been developed and currently the most accurate determination of *T. foetus* positive bulls is through collection and testing of preputial smegma for nucleic acid through employing a direct reverse-transcription quantitative real-time PCR (direct RT-qPCR) utilizing smegma [30]. The specific test developed by Ginter Summarell et al. eliminated the need to use culture medium. The direct RT-qPCR exhibited 100% diagnostic sensitivity and 100% specificity, whereas the currently employed qPCR (culture PCR), which utilizes cultured samples, exhibited 95% diagnostic sensitivity and 100% specificity [30]. Agreement between direct RT-qPCR and culture qPCR was found to be 98. Moreover, direct RT-qPCR identified three more positive samples and exhibited lower quantification cycle (Cq) values among positives by culture reading than did culture qPCR (direct RT-qPCR Cq range = 14.6–32.3 vs culture qPCR Cq range = 18.7–37.4) The direct RT-qPCR enables simplified sample collection, elimination of culture medium, faster results, applicability in cows, and lower cost than culture qPCR, making this new test more efficacious for control programs.

Treatment and Vaccination

Although there is no approved treatment for trichomoniasis in cattle in the USA, a study by Love et al. from 2017 suggests that treatment with metronidazole at a dose of 60 mg/kg of 0.5 μg/ml resulted in elimination and prevention of new growth of *T. foetus* [31]. The study was performed on a bull that tested positive for *T. foetus* on culture for five consecutive weeks. After treatment with intravenous metronidazole at a 24-hour interval for two treatments, the bull tested negative on culture for five weeks post treatment [31]. Although these results are promising, PCR was not included in this study, so further testing to confirm the ability of metronidazole to eliminate infection with *T. foetus* in cattle is likely warranted. Also, it must be noted that the use of drugs from the nitroimidazole family, which includes metronidazole, is banned in food animals in the USA. In human medicine, metronidazole is utilized for treatment of *Trichomoniasis vaginalis*. However, approximately 5% of strains of *T. vaginalis* in humans are resistant to metronidazole [32]. Additionally, metronidazole is an important antibiotic in the treatment of *Clostridium difficile* infections in humans and anything that will potentially add resistance to this serious toxin-forming bacteria is most likely not going to be taken off the prohibited list [32–35]. culture trails have been promising and *in vivo* trials are in progress to identify approved food-animal drugs that might be utilized for treatment of trichomoniasis.

At this time there is only one United States Department of Agriculture (USDA) approved vaccination for *T. foetus* in the USA, TrichGuard® (Boehringer Ingelheim). TrichGuard is a killed vaccine that requires two doses, administered two to four weeks apart and at least four weeks before the breeding season begins. This vaccination when administered correctly has been proven to decrease the severity of clinical signs associated with *T. foetus*. Additionally, vaccination against *T. foetus* decreases the time it takes females to clear the infection, thus returning them to cyclicity more quickly. Vaccination for *T. foetus* has been shown to promote production of vaginal, cervical, and uterine immunoglobulin (Ig) G1 and IgA antibodies in cows [16]. Cows able to clear the infection experience a transient resistance to reinfection [16]. In a 2017 study by Edmondson et al. 40 beef heifers were inoculated with either TrichGuard (20 heifers) or a sterile saline sham vaccination (20 heifers). The heifers underwent an estrous synchronization protocol and were inoculated vaginally with cloned *T. foetus*. Three days post-inoculation the heifers were maintained with three *T. foetus* negative, unvaccinated bulls for a 49-day breeding season. Cervical mucus samples were taken at designated dates and *T. foetus* positive cultures were obtained in all the heifers. Pregnancies were seen in both the TrichGuard (95%) and the sham vaccination group (70%). Heifers from both groups gave birth to live calves, with the TrichGuard group and the sham vaccination group only having a 50 and 20% live calf rate, respectively. Embryonic loss was observed in both groups, with the sham vaccination group experiencing a significantly higher loss (71%) versus the TrichGuard group (41%) [17]. Although the vaccine does not prevent infection with *T. foetus*, these results suggest that vaccination can significantly decrease the embryonic and fetal loss seen with *T. foetus* infections [17]. Two studies showed that 80% of vaccinated heifers challenged with *T. foetus* were negative within two weeks after a 15-day breeding cycle, as compared to 80% of non-vaccinated heifers still infected four weeks after a 15-day breeding cycle; in addition, 60% of non-vaccinated heifers remained infected 10 weeks following a 15-day breeding cycle [36, 37]. Hence, a vaccinated cow or heifer that is subsequently infected with *T. foetus* will clear the infection sooner and either maintain her pregnancy or be ready to rebreed weeks or months sooner than a non-vaccinated animal. Vaccination of bulls has been more closely evaluated in studies by Cobo et al. who found that immunoglobulins directed against *T. foetus* appeared in the preputial secretions of bulls vaccinated with *T. foetus* antigens [38, 39]. These and other studies

have evaluated the cellular and antibody response in the lower genital tract of bulls [38, 39]. These studies demonstrated that TrichGuard prevented colonization of the preputial and penile epithelium [38, 39]. In well-managed herds with firm vaccination protocols or herds that have experienced previous trichomoniasis outbreaks, vaccination is recommended.

Control and State Regulations

As there is no current legal treatment in the USA, many states have regulations in place for the movement and sale of bulls into, through, and out of their states. The current regulations in many states require bulls, virgin or non-virgin, that are greater than 18 months of age to test negative for *T. foetus* within 30 days prior to entering the state. In Texas bulls must be tested if they are 12 months of age or older. The bulls must be negative on PCR prior to entry into the state. If the bull is allowed to commingle with other cattle between the time of testing and the time of entering the state, the bull must be retested. The bull must be identified via a USDA issued ear tag, a registry brand, or registry tattoo. In addition to verifiable identification, the bull must have a current Certificate of Veterinary Inspection (CVI) that includes the test results, date, lab used for testing, address of both the consigner and consignee, and the signature of the accredited veterinarian associated with the CVI. Specific incidences may exempt animals from *T. foetus* testing; these include rodeo and exhibition bulls that will not commingle with cattle during their temporary stay in state and will leave the state immediately after the event. Also exempt are virgin bulls that are less than 18 months of age (but have CVI confirming age), bulls going directly to slaughter, and bulls that are strictly being transported through the state and will not commingle with female cattle. Every state has specific regulations regarding *T. foetus*, so it is imperative to check the requirements of shipping cattle to the particular state of interest at least two weeks prior to shipment.

Impact on Other Species

In addition to cattle, *T. foetus* can cause chronic infections in felines, with the predilection site being the gastrointestinal tract [4]. Colonization of *T. foetus* can occur in the stomach, cecum, and nasal passages of swine with no apparent clinical significance [40]. Although colonization in swine can occur, a study by Mueller et al. suggests that transmission between bovine and swine via swine fecal environmental contamination is unlikely [40]. In cats, *T. foetus* infections are characterized as large bowel diarrhea, with the organism colonizing on the mucosal surface of the colon [40, 41]. A study in 2007 by Stockdale et al. compared the effects on the reproductive tract of heifers experimentally inoculated with *T. foetus* of bovine or feline origin. This study revealed varying degrees of pathology in both the feline and bovine groups, suggesting that regardless of the organism's origin the same pathologic lesions are seen [41]. The largest difference in the pathologic lesions were seen in the heifers inoculated with bovine *T. foetus*, which were all devoid of an intact endometrial surface epithelium, whereas only one of the feline *T. foetus* inoculated heifers experienced endometrial epithelium damage [41]. The variation of the damage of the endometrium is suggestive of differing parasite interactions with the host [41]. This variation in the endometrial effects also suggests that feline *T. foetus* may not be associated with infertility in the bovine. The same study confirmed that although similar, feline *T. foetus* is not identical to *T. foetus* associated with bovine trichomoniasis [41].

Summary

Trichomoniasis is a venereally transmitted disease that can be economically devastating to a naïve herd. A bull infected with *T. foetus* is typically asymptomatic and becomes a chronically infected carrier. However, the cow experiences a transient infection manifested as embryonic loss and abortion and can develop immunity to *T. foetus*. The current standard of control is the identification and immediate removal of *T. foetus* positive bulls and prevention of exposure of infected animals to uninfected animals. Currently there are no approved treatments available in the USA. There are experimental treatments with promising results. Eradication is unlikely, but control with regular testing and strict biosecurity measures is vital to the success of prevention of further spread of trichomoniasis in cattle.

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Infectious Agents: Leptospirosis

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Introduction

Leptospirosis is a zoonotic bacterial disease considered to be an important cause of bovine reproductive failure worldwide. Caused by a motile, Gram-negative, obligate aerobic, spirochete bacteria in the genus *Leptospira*, infections may result in one or more of several different disease outcomes. In ruminants, disease forms may include relatively benign, chronic infections of the renal tubules and genital tract, or severe, acute disease of the renal, hepatic, or central nervous system, and reproductive inefficiency or abortion [1].

The taxonomy and system of classification of organisms in the genus *Leptospira* is complex and based on both genetic and serologic characteristics. Understanding the taxonomy of *Leptospira* requires familiarity with the hierarchical nature and meaning of terms such as serovar, serotype, and serogroup. Such terms have become necessary when describing leptospires due to the magnitude of both antigenic and genomic diversity found within the genus *Leptospira*.

The current standard for determining species of *Leptospira* is DNA sequencing and currently 20 different genomospecies (e.g. *Leptospira borgpetersenii*) have been identified [1–3]. The term serovar denotes an intraspecies classification of organisms based on serologic characteristics such as similar surface antigen expression [1, 3]. Serovars have been suggested to be the basic systematic unit of *Leptospira* spp. classification, with over 250 pathogenic serovars described [1–3]. Several different species are now known to include both pathogenic and non-pathogenic serovars. Each serovar may occur in more than one species (e.g. the serovar Hardjo occurs in the species *L. borgpetersenii*, *Leptospira interrogans*, and *Leptospira meyeri*) [2, 4]. Further differentiation among serovars is accomplished by serotype (e.g. *L. borgpetersenii* serovar Hardjo type hardjo-bovis). Serogroups are often used to group serovars with homologous antigen expression. Serogroups have no official taxonomic status, but are often useful for epidemiological studies involving similar populations or geographical regions [1–4]. Evidence exists for horizontal transfer of genetic material responsible for characteristics such as lipopolysaccharide (LPS) surface antigen expression between species of *Leptospira*, meaning neither serovar nor serogroup is currently predictive of the species to which an individual leptospire will belong [3, 4].

Although there are numerous species, serovars, serotypes, and serogroups within the genus *Leptospira*, the focus of this chapter will be on those most commonly implicated in reproductive inefficiency and failure in cattle.

Epidemiology

Prevalence

Members of the genus *Leptospira* are thought to be the most widespread zoonotic pathogens in the world [2]. Endemic serovars vary depending on geographical location; however, numerous studies have shown serovar Hardjo to be the most commonly isolated leptospire from cattle in the USA [5–8]. Two genetically distinct types of serovar Hardjo exist: *L. borgpetersenii* serovar Hardjo type hardjo-bovis and *L. interrogans* serovar Hardjo type hardjoprajitno. Serovar Hardjo type hardjo-bovis has a nearly global distribution [7, 9], while type hardjoprajitno is primarily found in the UK, and has not been identified in the USA [6].

Limited studies in the USA estimate herd prevalence of serovar Hardjo to be near 60% in dairies [10] and 40% in beef herds [6]. In Ontario, Canada, 8% of dairy herds and 44% of beef herds surveyed that were not vaccinated against leptospirosis were found to have cows serologically positive

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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for serovar Hardjo [11], whereas in western Canada seroprevalence appears to be much less in the beef herds surveyed there [12]. Herd prevalence may vary regionally, with higher prevalence more likely in temperate climates [6, 13]. In a large survey of cull cows sampled at slaughter in 49 states, the percentage of cattle serologically positive for a *Leptospira* serovar was much higher in southern tier and west coast states than other areas of the USA [5]. In studies of individual adult cows, seroprevalence of serovar Hardjo ranges from 10 to 30% in both the USA and Canada. Higher seroprevalence rates can be expected in individual herds that are endemically infected. One study performed in New Zealand of 95 beef herds totaling 1886 cattle found herd-level seroprevalence of serovar Hardjo to range from 79 to 90.5%, depending on whether the seropositive for a herd was defined as having one, two, or three animals seropositive in the herd [14]. A similar study conducted by Irish researchers found a beef herd-level prevalence of serovar Hardjo to be 82.29% over 288 herds [15]. These studies illustrate that broad variations exist in the seroprevalence of serovar Hardjo, and that these variations are likely due to differences in environment, management, and production system.

In the USA, serovars Hardjo, Pomona, and Grippotyphosa have been reported as the most prevalent in renal infections of cull cows [5]. Other serovars such as Canicola, Icterohaemorrhagiae, and Bratislava have been identified as causal agents in individual cases of bovine reproductive failure as well. A 2018 study conducted at harvest of fed cattle (<30 months of age) showed that 7.2% of 600 animals sampled were actively excreting leptospires in their urine prior to harvest. Of those, serovar Hardjo was the most commonly isolated, followed by Bratislava, Icterohaemorrhagiae, Grippotyphosa, and Pomona. Historically, exposure to serovar Bratislava among US cattle has been considered rare in occurrence. Although limited in scope, this study suggests serovar Bratislava may be increasing in prevalence [8].

Risk factors for individual animal and herd-level infections with *L. borgpetersenii* serovar Hardjo have been reported [6, 14, 16, 17]. Differences in risk factors reported among studies are likely due to variations in geographical region, environment, management, production system, etc. Although specific risk factors differ among studies, similar factors associated with *Leptospira* infection may be observed. Herd size has consistently been related to presence of *Leptospira* within a herd, with larger herds having greater odds of being seropositive for serovar Hardjo [14, 16, 17]. Other variables implicated in risk of seroprevalence include length of breeding season, higher mean annual temperatures [6], vaccination status [14], access to bodies of water [6, 14, 17], feeding on pasture, and no veterinary involvement [17]. Factors that should be considered when managing leptospirosis outbreaks include population size and density, environment, management system, and production goals.

Maintenance Versus Incidental Hosts

Animals infected with *Leptospira* can be classified as either maintenance hosts or incidental hosts of *Leptospira*. Different *Leptospira* serovars are associated with one or more maintenance host(s), which serve as reservoirs for transmission. Animals may be maintenance hosts of some serovars but incidental or accidental hosts of others. In a maintenance host, the disease is maintained by chronic infection of the renal tubules [2]. Transmission of the infection among maintenance hosts is efficient and the incidence of infection is relatively high at a population (or herd) level. Incidental hosts may become infected by direct or indirect contact with the maintenance host and are not important reservoirs of infection. Transmission of the infection from one incidental host to another is inefficient and uncommon. Examples of important maintenance hosts for different *Leptospira* serovars that commonly cause infection in cattle are listed in Table 59.1.

The terms "host-adapted" and "non-host-adapted" may often be used to describe infections or serovars of *Leptospira* in bovine hosts. Serovar Hardjo, including both types hardjo-bovis and hardjoprajitno, is the bovine host-adapted serovar, meaning the organism is adapted to surviving and reproducing within the bovine maintenance host. Maintenance infections in cattle would therefore by definition involve a host-adapted serovar, while incidental infections would involve a non-host-adapted serovar. Typically, host-adapted infections, or maintenance host infections, result in colonization of the urinary and reproductive tracts causing a chronic infection with no or mild clinical disease. In contrast, *L. interrogans* serovars Icterohaemorrhagiae, Bratislava, Canicola, Pomona, and Grippotyphosa are host-adapted to other species (see Table 59.1). Thus, when infections with these serovars occur in cattle they are described as non-host-adapted infections. Non-host-adapted infections in cattle may be more severe, but are often shorter in duration. The consequences of *Leptospira* infection in cattle may differ depending on if the serovar of *Leptospira* is host-adapted to cattle or another species.

Transmission

Both direct and indirect methods of transmission are important in *Leptospira* infections of cattle. Direct methods of transmission most often occur in infections involving

Table 59.1 Important maintenance hosts for different *Leptospira* serovars that commonly cause infection in cattle in the USA.

Leptospira species	*Leptospira* serovar	Maintenance host
L. borgpetersenii	Hardjo type hardjo-bovis	Cattle, deer [1, 9]
L. interrogans	Hardjo type hardjoprajitno	Cattle [1]
L. interrogans	Bratislava	Pigs, mice, horses [1, 2, 10]
L. interrogans	Canicola	Dogs [1, 2, 10]
L. interrogans	Grippotyphosa	Raccoons, opossums, skunks [1, 2, 10]
L. interrogans	Icterohaemorrhagiae	Rats [1, 10]
L. interrogans	Pomona	Pigs, raccoons, opossums, skunks [1, 2, 10]

serovar Hardjo, while indirect transmission most commonly occurs in incidental infections of cattle involving other serovars [18].

Direct transmission of bovine host-adapted serovar Hardjo occurs when uninfected cattle are directly exposed to contaminated urine, postpartum or postabortion uterine secretions, semen, or fetal membranes. Vertical transmission of serovar Hardjo may also occur, resulting in the birth of weak, *Leptospira*-infected calves. Shedding of leptospires from the urinary and reproductive tract may occur for months to years post infection with serovar Hardjo [1, 9, 18–20].

Indirect transmission is more prominent in non-serovar Hardjo infections. Bovine infections with non-host-adapted serovars of *Leptospira* such as Pomona, Icterohaemorrhagiae, and Grippotyphosa often result from environmental contamination with urine of other maintenance hosts such as rats, pigs, etc. Bovine non-host-adapted serovars often enter the environment through the urine of their maintenance host and survive until contact with a bovine incidental host occurs. Environmental contamination is often the cause of non-serovar Hardjo infections in cattle, rather than direct transmission from their maintenance hosts [18]. *Leptospira* organisms are able to survive in the environment for extended periods of time, particularly in warm and wet climates, and have been reported to survive in pond water for up to six months [7]. However, leptospires do not survive in hot and dry or freezing conditions. Cattle infected with non-host-adapted serovars typically shed low levels of the organism for a shorter time of weeks to months compared to infection with host-adapted serovars [1, 9].

The bovine host-adapted serovar Hardjo is very efficiently spread both directly and indirectly within a herd. Colonization of the urinary and reproductive tracts leads to continual shedding of the organism into the environment. Continual shedding lowers the importance of favorable environmental conditions, such as ponds and swampy areas, to maintain the infection in the herd and allows transmission to occur in confined housing or pasture situations [7, 18].

Pathogenesis

Leptospira organisms enter the body by penetrating mucous membranes of the eye, mouth, nose, or genital tract or through abrasions in the skin. Local proliferation at the site of entry occurs during an incubation period of 2–20 days. As the *Leptospira* organism proliferates, it enters the blood stream, leading to multiorgan infection, including colonization of the urinary and genital tracts. Leptospiremia typically lasts 7–14 days, during which signs of acute clinical disease may be present. Circulating antibodies against *Leptospira* organisms may reach peak levels at three to six weeks post infection. Antibodies can be detected in serum soon after leptospiremia begins and coincides with the clearance of *Leptospira* from blood and most organs. Upon cessation of the leptospiremic phase by *Leptospira* neutralizing antibodies, a convalescent phase begins in which low titers may be detectable for years in some animals. The humoral response is effective in clearing *Leptospira* organisms from most tissues in the body; however, colonization of the mammary gland, kidney, and gential tract continues as the organism appears to be protected from the immune response in these locations [1, 9]. Organisms can persist in the kidneys for several weeks in an incidental host or for six months or more in a maintenance host [7, 10]. *Leptospira* can also persist in the genital tract of a maintenance host for similar periods of time [10]. *Leptospira* excretion in urine is variable and may last weeks in incidental hosts and months in maintenance hosts. In cattle, serovar Pomona shedding has been observed for up to 15 weeks under experimental conditions [21], while serovar Hardjo is typically shed for 6–8 months [22] but has been reported to be as long as 18 months [23]. Adverse effects on reproduction in cattle may therefore be observed during the acute leptospiremic phase of disease due to invasion of the reproductive tract or systemic illness, or during the convalescent phase due to the lingering colonization of the genital tract.

Table 59.2 Summary of clinical differences between infections with Hardjo and non-Hardjo serovars.

	Hardjo serovar	Non-Hardjo serovar (e.g. Pomona, Grippotyphosa)
Presentation in adult cows	Subclinical to mild signs	Subclinical to mild signs
Persistence of infection	6–18 months	<6 months
Infertility	Common	Uncommon
Abortion	Occurs with initial infection	Occurs with initial infection
Time to abortion after infection	Months after infection	Weeks after infection
Herd presentation of abortion	Sporadic abortions	Sporadic abortions or abortion storms
Stillbirths	Yes	Yes
Weak calves at birth	Yes	Yes
Persistent infection in newborn calves	Yes	Yes

Clinical Disease

Clinical outcomes of *Leptospira* infections in cattle depend primarily on the serovar involved. Differences in the clinical manifestation of disease may be seen for bovine infections involving host-adapted serovars (e.g. Hardjo) when compared to non-host-adapted serovars (e.g. Pomona, Grippotyphosa). Level of host immunity may also influence the consequences of infection in cattle. A summary of clinical reproductive differences between bovine infections involving Hardjo and non-Hardjo serovars is presented in Table 59.2.

Infections with serovar Hardjo are typically subclinical or result in mild clinical signs. Clinical signs observed with serovar Hardjo infections are the result of persistent or long-term infection of the urinary and reproductive tracts. The most significant outcome of serovar Hardjo infection is decreased reproductive efficiency. Initial infections with serovar Hardjo during pregnancy can result in abortion, stillbirth, or birth of weak calves. Abortions may occur many weeks after infection of the dam and are usually not associated with any obvious illness in the cow. Infected but apparently healthy calves may also be born [20]. Abortions due to serovar Hardjo infection tend to occur sporadically as opposed to abortion "storms" which may occur as a result of infection with other non-Hardjo serovars such as Pomona or Grippotyphosa.

Persistent infection of the reproductive tract with serovar Hardjo can lead to infertility and early embryonic death. In infected herds, increased services per conception and prolonged calving interval have been described [18, 19, 24–26]. Studies in other species suggest that inflammation altering the uterine environment and direct invasion of the embryo by the *Leptospira* organism may be mechanisms by which persistent infection of the reproductive tract by serovar Hardjo decreases reproductive efficiency in cattle [27].

Infections with non-Hardjo *Leptospira* serovars are typically subclinical in adult cattle. If infection occurs during pregnancy, it can result in abortion, stillbirth, or birth of weak calves. Abortions are more common with non-Hardjo serovar infections. Abortion of autolyzed fetuses typically occurs one to four weeks after infection and without any overt clinical signs in the dam. Infections with non-host-adapted *Leptospira* serovars in calves, however, may result in severe systemic disease, including hepatitis, nephritis, and meningitis. Some serovars (i.e. Pomona and Icterohaemorrhagiae) produce hemolysins that result in acute intravascular hemolysis, leading to anemia, hemoglobinemia, and hemoglobinuria [1, 9].

Diagnosis

Accurately diagnosing leptospirosis as the cause of reproductive failure or inefficiency can be challenging for the practitioner and the producer. The effects of leptospirosis may not be observed for weeks to months after infection, such as in cases of estrus repetition or early embryonic death leading to an extended calving season or open cows. In other instances, producers may observe evidence of abortion (i.e. aborted fetus or fetal membranes) only after the decomposition process has begun, or they may not notice evidence of abortions at all. Even in cases of sudden "abortion storms," diagnosing leptospirosis as the definitive cause can be difficult.

Diagnosing leptospirosis as the cause of reproductive failure requires the practitioner to assess the clinical history of the reproductive-related complaint, clinical signs apparent within the individual or herd, underlying risk factors contributing to disease, and potential biosecurity risks (recent herd additions, exposure to wildlife, etc.). Critical evaluation of diagnostic testing results is also necessary to identify potential causes of false positives (vaccine type

and timing, etc.) or false negatives (sample contamination, improper sample handling, etc.). In cases where leptospirosis is suspected to be the cause of reproductive failure in a herd, risk factors that may have led to increased exposure to the organism or increased susceptibility to infection should be evaluated judiciously. Depending on the test performed, a positive result may only indicate carrier status and may be unrelated to the clinical problem. All diagnostic testing results should be interpreted within the context of the case history and the results of a thorough investigation into other potential risk factors for leptospirosis and reproductive failure. A summary of commonly used diagnostic testing methods for leptospirosis in cattle is presented in Table 59.3.

When investigating cases of suspected *Leptospira*-related reproductive failure, diagnostic testing methods for *Leptospira* involve detecting an immunological response to the organism (i.e. antibodies), or detecting the *Leptospira* organism or its DNA in tissues or body fluids of cattle [19]. Advantages and disadvantages exist for both schemes of testing; therefore utilizing a combination of testing modalities offers optimal diagnostic sensitivity and specificity [19]. Testing methods for serovar Hardjo focus on determining infection status of the herd. Repeated sampling is often needed to increase test sensitivity because of intermittent shedding in carrier hosts. Assuming a typical prevalence of 20–30% in herds endemically infected with serovar Hardjo, approximately 15 individuals would need to be tested to detect one infected individual with 95% confidence [32].

Serology is a commonly used and widely available method of *Leptospira* diagnostic testing that is inexpensive and reasonably sensitive [19]. The standard serologic assay is the microscopic agglutination test (MAT). The MAT involves mixing appropriate dilutions of serum with live *Leptospira* organisms belonging to relevant serovars found in the particular country or region where the testing is occurring [9, 19, 30]. If at the time of testing, high titer levels are observed with concurrent clinical disease and history consistent with leptospirosis, a diagnosis may be made. When cattle experience abortions as a consequence of infection with non-host-adapted serovars (i.e. Pomona, Grippotyphosa, Canicola, or Icterohaemorrhagiae), agglutinating antibody titers in the dam often exceed 1:1600. However, cattle infected with serovar Hardjo often show a poor antibody response following infection (i.e. titers ≤1:100), and at the time of abortion, serovar Hardjo titers may be low or negative [19].

Cross-reactivity among *Leptospira* serovars is not uncommon and can create confusion regarding the infecting serovar in individual animal tests. Cross-reactivity occurs when antibodies produced by an animal in response to infection by a particular serovar react with other serovars during the MAT. These cross-reactions may be equal to or higher than the reaction with the infecting serovar [9, 19]. Interpretation of serology may be complicated in animals vaccinated for *Leptospira*, as vaccines may induce a low-level antibody response that is detectable by MAT. In contrast to the high titers seen in natural infections, cattle typically develop titers that range from 1:100 to 1:400 in response to vaccines and these titers may persist for one to three months [19]. False positives are not common with MAT, as *Leptospira* surface antigens are not commonly shared with other organisms [29].

Table 59.3 Summary of commonly used diagnostic tests for detection of leptospirosis in cattle.

Test	Sensitivity	Specificity	Sample	Best use	Serovar differentiation
Detecting immune response					
MAT	Decreases as time from infection increases [9]; poor for serovar Hardjo [28]	Good [29]	Serum	Herd screening for exposure [30]	No [30]
ELISA	Depends on antigen used in test preparation [9, 30]	Depends on antigen used in test preparation [9, 30]	Serum	Herd screening for exposure or individual case confirmation [30]	Yes (LPS-based test) [9, 30]
Detecting *Leptospira* agent					
Culture and identification[a]	Poor due to fastidious growth requirements [29, 30]	Good [29, 30]	Urine, fetal tissue, and fluid	Individual case confirmation [30]	Yes [30]
PCR	Good (decreased by contamination) [30]	Exquisite [30, 31]	Urine, fetal tissue, and fluid	Individual case confirmation [30]	No [9]

[a] Isolate identification following culture requires a specialized reference laboratory and is beyond the scope of this text.

Serology is most sensitive when diagnosing the acute phase of leptospirosis, the sequelae of which is often abortion in cattle [9]. Because antibody titers are often highest at the time of abortion and may persist for months, paired serology performed post-abortion often does not demonstrate meaningful differences in titer levels. Paired serology initiated prior to abortion would likely demonstrate noticeable changes in titer levels; however, abortions may be the only clinical sign observed by the producer. The sensitivity of serology is less when serovar Hardjo infections are involved, because antibody titers are often low or absent. Serology is not appropriate when screening individuals for carrier status, because cattle chronically infected with serovar Hardjo may be seronegative while continuing to shed leptospires in urine [28]. Therefore leptospirosis should not be ruled out based on low antibody titers alone, especially when serovar Hardjo infection is suspected [19].

Enzyme-linked immunosorbent assays (ELISAs) offer the ability to detect antileptospiral antibodies following infection. The primary antigen preparations used for ELISA development include outer membrane protein (OMP) preparations and whole-cell preparations. Specificity of the ELISA is dictated by the antigen used. Recombinant OMP-based ELISAs produce reactions to numerous pathogenic leptospires and lack the ability to differentiate between *Leptospira* serovars. Cattle infected with serovar Hardjo produce little response to OMPs, making OMP-based ELISAs of no use in control programs where host-adapted infections are involved. LPS antigen-based ELISAs are capable of differentiating between serovars and are more useful for epidemiological investigations and herd control programs. ELISA tests are not useful in vaccinated herds, as *Leptospira* vaccines stimulate antibody production leading to false positive reactions. The MAT has historically been used to validate ELISAs. This method of validation is problematic, however, as the MAT can have sensitivities of 50% or lower in chronic *Leptospira* infections. Although ELISA-based testing may provide benefit in some situations, lack of availability of OMP-based ELISAs as well as concerns regarding test validation make ELISA-based testing impractical in most circumstances [9, 30].

Detection of *Leptospira* organisms or their DNA may be accomplished by immunofluorescence, immunohistochemistry, culture, and polymerase chain reaction (PCR) assays performed on tissues or body fluids. Often a combination of at least two of these techniques can be used to overcome issues with sensitivity that individual tests may encounter [33]. Immunofluorescence can be used to directly identify organisms within tissues such as fetal liver, lung, kidney, or placenta, or in urine sediment. *Leptospira* can be identified histologically by evaluation of formalin-fixed tissues if special stains are applied. When samples are available following abortion, fetal placenta, lung, liver, and kidney may be submitted for histopathological evaluation. Necropsy samples taken from adult kidney may also be useful in detecting *Leptospira* through histopathology [19].

Bacteriologic culture of *Leptospira* organisms is difficult due to the fastidious nature of its growth requirements; however, culture does provide a definitive method for the diagnosis of leptospirosis and identification of the infecting serovar. Culture may be performed on tissues harvested from necropsy of aborted fetal tissues or from urine voided from animals with clinical signs or suspected of having leptospirosis. Leptospires can typically be found in the urine of cattle for 10 days following the onset of clinical signs (i.e. abortion) [19]. Although culture of the organism provides near 100% specificity, the technical difficulty of the culture process for leptospires results in poor sensitivity [9]. Intermittent shedding in carrier hosts also lowers sensitivity of culture. Administration of the diuretic furosemide prior to urine collection increases the number of organisms present in the urine by increasing glomerular filtration rate, thereby improving the sensitivity of diagnostic methods [34]. Additionally, urine dilution enhances the survival of *Leptospira* for culture purposes [19]. A negative culture of urine or tissue should not be relied upon solely to rule out leptospirosis.

PCR is also available as a tool to aid in the diagnosis of leptospirosis and its use is increasing. The benefits of PCR include favorable sensitivity, the ability to detect early infections, and that it does not require a live organism. Variations in procedure between laboratories may affect the accuracy of PCR [33]. A positive PCR indicates the presence of genetic material from pathogenic leptospires, but serovar identification is not possible with most methods [9]. Leptospires may be detected by PCR performed on tissue samples from the aborted fetus, but urine from the dam typically provides a more reliable result [19]. Performing PCR on the stomach contents of an aborted fetus may also aid in diagnosis of leptospirosis abortion [35]. As with any diagnostic test, inaccurate results may be encountered when using PCR. False negatives are often seen because tissue samples may contain inhibitors to the amplification process [19]. False positive results may occur from contamination with foreign genetic material, as complex PCR assays can be extremely sensitive [31]. Failure of PCR to detect organisms in urine does not rule out infection, as the animal may not have been shedding the organism when the sample was collected [9]. As with bacteriologic culture, furosemide can be used to increase leptospire numbers in urine [34].

Control

Given the widespread exposure to wildlife and domestic animals that may be carriers of leptospirosis and the high prevalence of serovar Hardjo infection in cattle, prevention of all exposure to leptospirosis is virtually impossible in most dairy and beef operations. Therefore control of leptospirosis in cattle requires a comprehensive approach that includes reducing the risk of *Leptospira* exposure by interrupting direct and indirect routes of transmission, vaccination, and selective antimicrobial treatment to clear carriers of serovar Hardjo when indicated.

Limiting direct and indirect contact between cattle and carriers of *Leptospira* should be a priority. Strategies would include controlling wildlife and domestic species known to be maintenance hosts of serovars that commonly infect cattle (Table 59.1). Strategies should focus on reducing access of both wild and domestic animals to cattle feeds, especially stored feeds such as hay, grain, and other commodity feedstuffs. Control measures should also attempt to limit cattle access to standing surface water such as swamps or ponds to which wildlife commonly have access. Quarantine procedures can be implemented to reduce the risk of introducing serovar Hardjo into a herd through purchase of infected cattle. During quarantine, antimicrobial treatment and vaccination can be used to eliminate convalescent serovar Hardjo infections in new cattle and reduce the risk of reinfection and shedding.

Vaccination is commonly practiced in the USA to prevent leptospirosis. Currently available vaccines appear to be effective in limiting the shedding of serovar Hardjo in urine [36]. Whole-cell bacterins containing serovars Pomona, Canicola, Icterohaemorrhagiae, Grippotyphosa, and Hardjo ("five-way" vaccines) are widely available and commonly used. In general, these vaccines provide good protection against disease induced by each of the serovars, except serovar Hardjo [20, 37]. New-generation leptospirosis vaccines specifically targeting serovar Hardjo have demonstrated superior ability to prevent renal infection, urinary shedding, and fetal infection [27, 38]. Single dosing regimens and dosing regimens that included a booster four months after the initial dose have both proven to create a significant serologic response and prevent renal colonization [39]. These new monovalent vaccines induce a strong and long-lasting cell-mediated immune response compared with cattle naturally infected with serovar Hardjo and cattle vaccinated with non-protective serovar Hardjo vaccines [40, 41]. A general vaccination strategy for leptospirosis would include a whole-cell bacterin directed against serovars Pomona, Canicola, Icterohaemorrhagiae, and Grippotyphosa and a new-generation vaccine directed against serovar Hardjo. Recent studies, however, indicate that concurrent use of new-generation monovalent vaccines for serovar Hardjo used in conjunction with systemic oxytetracycline did not improve reproductive performance in dairy [42] or beef [43] herds where traditional "five-way" vaccines were already being used. The herds in these studies were not experiencing reproductive losses, however, so in instances where serovar Hardjo is suspected to be a source of reproductive inefficiency, a new-generation monovalent vaccine is recommended. Immunity should be induced in replacement breeding cattle following the manufacturer's recommendations and then revaccination is recommended every 6–12 months.

Antimicrobials can be used to treat cattle harboring persistent serovar Hardjo infections, as well as those with non-host-adapted *Leptospira* infections. Currently, there are no antibiotics approved for treatment of leptospirosis in dairy or beef cattle in the USA. Antibiotic selection for extralabel treatment of leptospirosis should consider potential milk and meat withdrawal times. Antibiotic treatment stops urinary shedding and is likely to improve clinical signs associated with persistent colonization of the reproductive tract with serovar Hardjo. Long-acting forms of oxytetracycline, tilmicosin, tulathromycin, and ceftiofur have been shown to be effective in the treatment of serovar Hardjo infections [44, 45]. One study showed that a single dose of tulathromycin (2.5 mg/kg, SC) resulted in clearance of leptospires from the urine, kidney, oviduct, and uterus as demonstrated by culture and PCR [45]. Another study found that a single dose of oxytetracycline (20 mg/kg, IM) resulted in no evidence of urinary shedding detected by PCR, culture, or immunofluorescence [44]. Caution should be exhibited when using any antimicrobial at extralabel doses and the Food Animal Residue Avoidance Databank (FARAD) should be consulted for appropriate withdrawal times. Antimicrobial treatment should be combined with vaccination to prevent reinfection from occurring.

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Infectious Agents: Brucellosis

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Introduction

Brucellosis is a zoonotic disease caused by various species of *Brucella*, a Gram-negative facultative intracellular bacterium [1]. The disease severely impacts human health and livestock productivity in much of the world, especially in low-income countries, which has led the World Health Organization (WHO) to classify brucellosis as one of the world's top "neglected zoonotic diseases" [2]. While animals are the primary hosts of brucellosis, the disease is readily transmitted to humans primarily through the consumption of unpasteurized milk and dairy products and by direct contact with infected animals [3].

Evidence of brucellosis has been present for centuries, with the first recorded descriptions of disease characterized by fevers in 1859 [4]. The disease was discovered in Malta, where it was infecting British military officers and the local population. A researcher, Dr. David Bruce, traveled to Malta and was able to culture the bacteria causing the disease, which was eventually called brucellosis [5]. Subsequently, Themistocles Zammit was able to determine the connection between infected goats on the island and the transmission of brucellosis, primarily through milk [5].

Brucellae have varying host preferences and pathogenicity [6]. The *Brucella* species of particular importance to humans and livestock worldwide include *Brucella abortus*, *Brucella melitensis*, and *Brucella suis*, whose primary hosts include cattle, sheep and goats, and swine, respectively [7]. All of these *Brucella* species, along with *Brucella canis* (dogs), are also pathogenic for humans [8]. Although brucellosis is primarily host specific, cross-species transmission can occur with enough exposure, and cattle can become infected with *B. suis* or *B. melitensis* when they share facilities or pastures with infected pigs, sheep, or goats [9].

In addition to the "classical" *Brucella* species, other species exist and continue to be discovered. *Brucella ovis* in sheep causes ewe abortion, epididymitis in rams, and lamb neonatal mortality [10]. *B. ovis* is not known to be a human pathogen and therefore is not routinely included in brucellosis eradication programs. *Brucella neotomae* is known to affect rodents, and is also not a human pathogen [11]. Novel species more recently discovered include *Brucella ceti* which affects porpoises, dolphins, and whales, and *Brucella pinniedialis*, which affects seals. Additionally, *Brucella microti* (red foxes, common voles) has been isolated. The significance of these novel species is unknown, although they have the potential to be human pathogens. None of these novel species is known to affect cattle [11].

The facultative intracellular characteristic of *Brucella* allows the organism to largely evade the host immune system [12]. When cultured, *Brucella* has either a smooth or rough colony morphology, based on the composition of the lipopolysaccharide (LPS) molecule, which is the major inducer of antibody responses [13]. The naturally rough types (*B. ovis* and *B. canis*) tend to have less virulence to other than their host species (sheep and dogs) than the smooth types more commonly associated with livestock [13]. The difference in compositions of the LPS molecule between the smooth and rough types plays a role in serologic diagnostic testing [13].

Brucellosis is considered one of the most economically important zoonoses globally, with economic impacts attributable to human, livestock, and wildlife disease [14]. In high-income countries such as the USA, brucellosis has been successfully controlled or eliminated in most livestock populations, yet may persist in wildlife populations such as feral swine or bison and elk. In low-income countries it is often endemic, with large disease and economic burdens on both human and animal populations [14].

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Etiology

Brucella abortus

B. abortus is the primary cause of brucellosis in cattle. There are seven biovars of *B. abortus* occurring in the world, the most predominant one being Biovar 1 [9]. *B. abortus* was initially isolated in 1895 from aborted materials from cattle by Bernard Bang, a Danish veterinarian [15]. As a result of that discovery, brucellosis in cattle is sometimes referred to as "Bang's disease" [16].

Cattle infected with *B. abortus* often exhibit placentitis and abortion [17]. *B. abortus* has a great affinity for the pregnant uterus, and often localizes in the reproductive tract of both male and female cattle [12]. Abortions in affected animals most commonly occur in the third trimester of gestation [18]. It has been determined that cows infected with *Brucella* are three to four more times likely to abort than non-infected animals [19].

Although the most common clinical sign of brucellosis in newly infected animals is abortion, the infected cow is often asymptomatic when not pregnant [19]. However, when such animals calve or abort, they become highly efficient transmitters of brucellosis via birthing materials and products of parturition [20]. Aborted fetuses and fetal membranes and fluids contain high levels of bacteria and therefore cause environmental contamination, resulting in a high risk of infection of susceptible animals [12]. Up to 10^{10} *Brucella* organisms per gram have been measured in placental samples from abortions caused by brucellosis [21]. Abortions may also be followed by long-lasting vaginal discharge, further leading to environmental contamination [22]. After contracting brucellosis, approximately 80% of cows normally abort only once. In subsequent pregnancies, infected cows may give birth to weak calves or may have apparently normal calvings [19]. However, when an infected cow calves, there is heavy shedding of bacteria through fetal fluids and the placenta, and such animals are sources of infection for other animals [19]. In between pregnancies, the *Brucella* tend to localize in lymph nodes and mammary glands [9].

Orchitis, epididymitis, and seminal vesiculitis may be seen in bulls, thus resulting in impaired semen production and infertility [12]. In cattle, natural breeding is not a major route of infection [23], although artificial insemination with contaminated semen may result in transmission to cows [12]. Hygromas and arthritis may also be seen in cattle [9].

Cattle most commonly become infected with brucellosis by ingestion of the *Brucellae* in contaminated pastures, feed, or fodder. Cows commonly lick newborn calves, afterbirth, and fetuses, all of which often contain a large number of bacteria and are major sources of infection [9].

The control of brucellosis in cattle is complicated by the role that heifers play in maintaining the disease in affected populations. Exposed heifer calves have been shown to be negative to serological tests, yet abort or have an infective first calving [24]. In addition, a small but important percentage of heifer calves born to seropositive dams have been found to be seropositive long after being isolated from potential other exposure. Those heifers are referred to as "latently" infected and are a risk for reinfection of herds [24]. The percentage of latently infected heifers has been estimated to be about 2.5% [25].

Brucellosis caused by *B. abortus* causes severe economic losses to cattle producers due to abortion losses, culling losses, and production losses [7]. It is estimated that brucellosis causes a 20–25% loss in milk production [9]. In addition, economic losses due to regulatory control and eradication programs requiring testing and removal of positive animals [26] and the impact of associated quarantines can be significant.

Brucella suis

Brucellosis in swine is caused by *B. suis*, which was first isolated by Traum from aborted swine fetuses in 1914 [12]. Swine brucellosis has a worldwide distribution, and both domestic and feral swine are hosts for *B. suis* [6]. In the USA, *B. suis* has essentially been eradicated from domestic commercial swine, yet remains endemic in feral swine [27]. The range of feral swine across the USA is expanding rapidly from its initial ranges of the southern part of the country [28]. As the expansion of feral swine continues, the risk of the spread of *B. suis* also increases.

Although swine can be transiently infected with other *Brucella* species, *B. suis* is the only *Brucella* species that can cause generalized systemic infection in pigs, resulting in reproductive failure [12]. In affected sows, abortion is most commonly observed and can occur at any stage of gestation, but most frequently between days 50 and 110 [18]. In herds that are chronically infected, infertility may be the most clinically apparent sign [18]. Other clinical signs in swine include orchitis in boars, posterior paralysis, and lameness [18, 29].

Transmission of *B. suis* usually occurs through direct contact with infected swine, and through the consumption of feed contaminated by abortion products or uterine discharges [18]. The boar is commonly incriminated as an important means of spread. In swine, unlike in cattle, brucellosis is a sexually transmitted disease and can be readily transmitted through breeding and the use of semen from an infected boar during artificial insemination [30].

Diagnosing brucellosis in swine is challenging in that the sensitivity and specificity of diagnostic tests in swine are

lower when compared to the detection of brucellosis in cattle, because serological tests used were developed for the detection of the *B. abortus* O side-chain in infected cattle [31]. In one study, a panel of serological tests conducted on infected feral swine only correctly identified 52% of those animals as infected [31]. In another study, 17% of culture-positive swine were negative on all serological tests [31]. Therefore serological tests for brucellosis in swine are usually interpreted at a herd level as opposed to an individual animal level [31].

Cattle grazing on pastures in close proximity with infected feral swine have also become infected with *B. suis* [32]. Although infection with *B. suis* does not result in abortion or decreased production in cattle, serologic titers of cattle infected with *B. suis* cannot be distinguished from those infected with *B. abortus* [32]. Therefore significant economic losses may result from an incident of such cross-species infection as a result of subsequent trace-back investigations, whole herd testing, and removal of positive animals required to definitely determine which *Brucella* is involved [32].

B. suis is also a serious human pathogen, with a higher degree of pathogenicity than *B. abortus* [29]. Therefore humans who have exposure to infected swine, such as hunters, wildlife biologists, and abattoir workers, are at an increased risk for transmission [29].

Brucella melitensis

B. melitensis is the causative agent of brucellosis in sheep and goats, and has significant impact on both the livestock industry and human health worldwide [33]. Of all the *Brucellae* known to affect humans, *B. melitensis* is the most virulent species, causing significant debilitating disease [33]. In 1905, a Greek physician was able to build an epidemiological link between the consumption of goat milk and "Malta Fever" in humans [33]. However, there is historical evidence that *B. melitensis* has been present for centuries [33].

Characteristic clinical signs of brucellosis in a sheep flock or goat herd are associated with an extensive negative impact on production, and include middle- to late-term abortion, stillbirths, and the birth of weak offspring [34]. The disease in males commonly causes impaired semen quality and therefore loss of fertility [12].

The USA is free of *B. melitensis*. However, in 1999, *B. melitensis* was discovered in Texas in four cows and traced back to an associated infected herd of meat goats, which was subsequently depopulated. That was the first time *B. melitensis* had been confirmed in a cow in the USA. The source of the *B. melitensis* infection was never definitively determined, but is thought to have been imported goats from Mexico [35].

Pathogenesis

The most common portal of entry for *Brucella* in the bovine is through the ingestion of aborted materials such as placentas and birthing fluids, by licking aborted fetuses or newborn calves from infected cows, or by ingesting feed or water contaminated by those products [19]. Once ingested, the *Brucella* invades the mucous membranes of the mouth, throat, and regional lymph nodes (suprapharyngeal and mandibular), where it localizes during the incubation period [20]. During the incubation period, *Brucella* replicates intracellularly in phagocytes [36]. This is followed by a brief bacteremia leading to systemic infection, with the bacteria localizing preferentially in the pregnant uterus, mammary gland, and male genital organs [36]. *B. abortus* thrives in the uterus during the last trimester of gestation. Although controversial, this is believed to be due to the high concentration of erythritol, which is metabolized by *Brucellae* as a source of energy and carbon [36]. The multiplication of *Brucellae* results in vasculitis, trophoblastic necrosis, infiltration of inflammatory cells, and ulceration of the allantochorion, thus compromising fetal–maternal metabolic exchanges and resulting in abortion [36]. *Brucella* bacteria also localize in the udders of animals, thus resulting in the production of contaminated, infectious milk [21].

Brucellae reside within and replicate within macrophages, dendritic cells, and trophoblasts [12]. The intracellular nature of *Brucella* organisms allows the bacteria to evade the host's cellular response and makes it difficult to eliminate by the use of antimicrobial drugs [37]. This allows for retention of the sequestered *Brucellae*, often resulting in subsequent relapse of infection [37].

Approximately 60–70% of calves born from infected dams are themselves initially infected. However, almost all recover within months of removal from the infective source and very young calves are known to be more resistant to infection. A small percentage of these exposed calves harbor latent infection yet remain negative to diagnostic tests until they abort their first pregnancy. These heifers are a significant threat to the success of control and eradication programs [19].

Susceptibility to brucellosis and the incubation period are quite variable, depending on factors such as age, pregnancy status, and sex of the animal, and the amount of bacteria to which the animal is exposed [25]. Although very young animals are relatively resistant to sustained infection with brucellosis, heifers that have reached sexual maturity increase in susceptibility, becoming extremely susceptible as they advance in pregnancy.

Immune Response

Exposure to *Brucellae* induces both cellular and humoral host immune responses. While host resistance to *Brucella* spp. is not completely understood, cell-mediated immunity appears to play a major role in the immune response to infection with virulent *Brucellae* [1]. This cell-mediated immunity involves activation of the bactericidal mechanisms of macrophages and dendritic cells and the expansion of antigen-specific T-cell clones [38]. The humoral response is limited and not protective, yet it is important from a clinical perspective in the detection of antibodies induced by *Brucellae* which are commonly used in serology for the diagnosis of brucellosis in humans and livestock [38].

As with other diseases, the innate immune system serves as the first line of host defense for brucellosis, responsible for preventing replication of the bacteria, reducing the initial number and killing of the bacteria, and creating conditions for generating an effective adaptive immune response [17]. Although there are countless components of the innate immune system to infection by *Brucellae*, they are not sufficient to overcome the infection [17]. The development of an adaptive cell-mediated immune response is necessary to control the disease [17].

An effective cell-mediated immune response against intracellular infections such as brucellosis is characterized by the predominance of a T helper type 1 (Th1) cell response that includes the secretion of interferon (IFN)-γ [17]. Activation of macrophages by IFN-γ is an important factor in the control of brucellosis [39]. Activated macrophages play a key role in the elimination of *B. abortus* [17, 39].

The exact contribution of humoral immunity in resistance to brucellosis probably plays a secondary role as it is also seen in other intracellular infections [17]. The major virulence factor of *Brucella* is the LPS in the outer membrane of the bacteria, which plays a role in cell entry and immune evasion [40]. The LPS O-side chain (OPS) is the primary immunogenic component of smooth *B. abortus* strains, and the majority of the antibody response in both human and animal infections is directed against the OPS antigen [17]. Brucellosis diagnostics are primarily based on serology, with agglutination tests being based on reactivity of antibodies against the LPS [40].

Diagnostics

The definitive diagnosis of brucellosis in livestock depends on laboratory testing, as clinical signs are not pathognomonic [6]. Laboratory diagnosis methods include serology, bacteriology (isolation of the bacteria), and molecular assays.

Unfortunately, there is no current diagnostic test sufficiently sensitive and specific enough to be capable of detecting all stages of infection in live animals [41]. The diagnostic "gold standard" is considered to be bacterial culture and isolation; however, that is not always practical in that samples are difficult to obtain, and isolation often is not successful due to the facultative intracellular nature of the bacteria. There is also a risk of infection to laboratory personnel [42]. The choice of samples for culture may include vaginal secretions, milk, colostrum, or mammary tissue, aborted fetuses (lung, stomach, or spleen), fetal membranes, and certain lymph nodes (oropharyngeal, scapular, iliac, and supramammary/testicular) [6]. However, because of the lack of sensitivity of most culture procedures and the associated drawbacks, serology is usually used for the diagnosis of brucellosis in animals [43].

The first agglutination test for the detection of antibody to *Brucella* was reported over 100 years ago [44]. Since then, diagnostic methods and accuracy have improved and new assays have been developed. However, the diagnostic sensitivity and specificity of serological tests vary widely, and there is debate in the literature over the value of different tests. Different serological tests measure different antibody classes [45], so several serological tests are usually used when making a diagnosis.

Serological tests are generally either primary binding assays, which directly measure the interaction between antibody and antigen, or the more conventional tests which measure secondary reactions, such as agglutination or activation of complement [44]. The primary binding tests, such as fluorescence polarization assay (FPA) and competitive enzyme-linked immunosorbent assay (C-ELISA), were developed to be more sensitive and specific than conventional agglutination tests [44], yet require specialized equipment or kits. The more conventional or secondary binding tests, such as the Card test, the Rose Bengal test (RBT), the rivanol test, the buffered acidified plate test (BAPA), and the complement fixation test (CFT), are dependent on being able to see the biological interaction of antibodies with antigen with the naked eye. These tests measure the consequences of antigen–antibody union such as agglutination, precipitation, or complement fixation.

Traditionally, highly sensitive, inexpensive, fast yet not necessarily highly specific serological tests (such as the Card test, RBT, or BAPA, and may include the FPA and ELISA) are used as initial screening tests [44]. Samples showing reactivity to screening tests are then subjected to confirmatory tests, which must be both sensitive and specific [46]. Such serologic tests may include the CFT, FPA, or ELISA. However, serological tests cannot determine which *Brucella* species is present in the host as only antibodies are measured.

Supplemental culture and isolation or DNA detection by PCR allows for a definitive diagnosis [6].

Once brucellosis is identified in livestock, state and/or federal animal health officials will become involved to control and eliminate the disease from the affected population(s).

Brucellosis in Wildlife

In the USA, brucellosis has been essentially eliminated from cattle. However, there are two primary reservoirs of *B. abortus* remaining: elk (*Cervus elaphus*) and free-ranging bison (*Bison bison*), both in the Greater Yellowstone Area (GYA – Yellowstone National Park and areas surrounding it) [27]. The risk of the spread of brucellosis between wildlife, livestock, and humans is a legitimate concern that creates serious challenges for wildlife management and conservation in those areas, especially near the boundaries of national and state parks and elk feeding grounds in the vicinity [47]. Although originally considered a spillover disease from cattle to bison and elk, brucellosis now regularly spills back from elk to cattle in the GYA. Bison to cattle transmission has not been documented in the GYA under natural conditions, most likely because of rigorous management to keep cattle and bison separated, although transmission has been demonstrated experimentally [47]. Transmission from bison to cattle has also been demonstrated when the species are pastured together [48].

B. suis is present in feral swine populations in the USA, although it has been essentially eliminated from domestic swine. The high incidence of *B. suis* in feral swine has resulted in transmission to domestic swine when raised in outdoor pens, and also to domestic cattle, causing serologic titers that are indistinguishable from those induced by *B. abortus* [31]. There is a risk to public health if raw milk is consumed from *B. suis* infected cattle, which are generally asymptomatic yet may shed high levels of bacteria in milk [31]. In addition, although *B. suis* does not result in abortion or decreased production in cattle, the detection of serologic titers as a result of *B. suis* infection causes economic losses incurred as a result of the subsequent disease investigation, including whole-herd testing, removal of infected animals, and trace-back investigations [49].

Brucellosis Regulatory Programs

Efforts to eradicate brucellosis in cattle in the USA were initiated in 1934 due to the Great Depression and concurrent drought conditions resulting in a need to reduce the number of cattle. At that time, brucellosis was the most significant livestock disease problem in the USA, with 11.5% of cattle tested in 1934–1935 determined to be infected [20]. In 1954, after recognizing the magnitude of the brucellosis problem and its impact on human health and the cattle industry, Congress appropriated funds for a national program to eradicate brucellosis from cattle [20]. As of 31 December 2000, for the first time, there were no brucellosis-affected cattle herds in the USA. As expected due to the variable incubation period, an occasional additional affected herd was disclosed after that time and quickly eliminated [20]. Currently, although there are occasional spillover cases from elk in the GYA to cattle, there have been no other cases of brucellosis caused by *B. abortus* in cattle in the USA since 2010 [50].

Surveillance

For a disease eradication program to be successful, an essential first step is to have an effective and ongoing surveillance system. It is imperative to be able to find, contain, and eliminate the disease before it spreads to other susceptible populations [20]. A recent review of the brucellosis control or eradication programs in 23 countries found that even if all the tools needed to eradicate brucellosis were available, an efficient brucellosis surveillance system is critical to program success [51]. Effective surveillance for brucellosis must be ongoing and utilize multiple methods [20]. In the USA, surveillance in the early stages of the brucellosis eradication program included down-the-road testing of herds. Later, the surveillance system was expanded to include the collection of blood samples at slaughter, testing of cattle at the first place they were assembled (such as stockyards and livestock markets), change of ownership testing, and milk ring testing of dairy herds. Additional surveillance methods included wide area testing in the vicinity of affected herds and testing of high-risk herds identified through epidemiologic investigations [20].

Since the USA has been essentially free of brucellosis for a number of years (other than occasional spillovers into cattle from elk in the GYA), national surveillance methods have recently been modified. Currently, around two million animals are tested annually at slaughter. This level of surveillance is sufficient to detect one affected herd out of 100 000 herds at a confidence level of 95%, with an estimated prevalence of 0.002%. This level is higher than what is required by the World Organization of Animal Health (OIE) and is intended to document disease-free states [50]. Federal or state animal health officials may also implement targeted surveillance activities as needed in areas determined to be high risk [52]

Control and Eradication Programs

Brucellosis is a regulated disease in the USA, meaning once a suspected case is identified, it is investigated by state or federal veterinarians. The US brucellosis eradication program historically consisted of several components, including serologic testing at the first point of assembly of cattle, trace-back of reactor animals to the herd of origin, quarantine of infected herds, test and removal of seropositive animals or whole herd depopulation, and vaccination of heifers [53]. Once brucellosis has been diagnosed in a herd, control measures are generally based on the principles of test and slaughter, vaccination, and sanitation [54]. In the USA, historically herds were tested every 30–180 days as part of a herd plan that also included herd management, until the herd was determined to be free of disease, and positive animals were removed to slaughter. As the country approached becoming free of brucellosis in domestic cattle, affected herds were generally depopulated [20]. However, in some cases and in some parts of the world, depopulation is not an option. In those cases, successful elimination of the disease depends on the development of a herd plan of action that will combine sound epidemiological practices with good animal husbandry practices, with the goal of eradicating the disease in the herd [55]. This involves far more than just blood testing the herd and removing reactor animals. Control and subsequent elimination of brucellosis from a herd or community is based on interruption of transmission, by minimizing exposure and the dose of bacteria to which animals are exposed. An effective herd plan should be designed to manage the disease problem and should include more than just a testing schedule. Its purpose is to eliminate the disease within the herd and to prevent reintroduction of disease into the herd. Since brucellosis is spread at the time of abortion or calving, management to prevent transmission during these times is essential. As pregnant animals are the ones that are most likely to continue spreading the disease, those need to be intensely managed [56]. In order to effectively control and eventually eliminate brucellosis from a herd and a community, calving management, heifer management, testing schedules, vaccination plans, and the management of replacement animals must all be addressed. Herds become infected with brucellosis through three primary ways: community spread from infected neighbors (including elk in the GYA), the introduction of incubating animals (tested or untested), and the retention of infected heifers. All three of those possible routes should be managed to prevent the reintroduction of brucellosis to negative herds.

Vaccination

The use of vaccine has been a critical component of most programs for the control or eradication of brucellosis in livestock caused by *B. abortus* or *B. melitensis*, but not for other *Brucella* spp. [54]. Vaccination has been a major factor in the success of the brucellosis eradication program in the USA. The ability of a vaccination program to reduce prevalence of brucellosis is generally dependent upon the proportion of vaccine-eligible animals within the targeted population that are vaccinated [54].

An ideal vaccine for brucellosis has been described as one that (i) does not induce antibodies that interfere with diagnostic serological testing; (ii) is attenuated and does not cause disease in animals or is not pathogenic to humans; (iii) does not lead to seroconversion with revaccination; (iv) is a live vaccine and able to provide a strong Th1 immune response; (v) is able to induce a strong and persistent protection against systemic and uterine infection; (vi) is stable and does not revert to virulence either *in vitro* or *in vivo* and (vii) is easy to produce and administer and inexpensive [57]. Although there have been research efforts toward developing killed vaccines, those vaccines have been found to not adequately stimulate cellular immune responses, and thus are not as efficacious as modified live vaccines [54].

However, there is no perfect vaccine for brucellosis, as no vaccine has been proven to provide 100% protection under field or experimental conditions [54]. The use of vaccination alone has been shown to reduce disease prevalence, but it is not likely to completely eliminate brucellosis from an infected herd. Exposure to high doses of *Brucella* can overwhelm protection from vaccine [54]. Although vaccination alone is insufficient to eliminate the disease from a herd, it plays an important role in that it increases resistance to infection and reduces both the risk of abortions and the number of bacteria shed by an infected animal [58].

In the USA, *B. abortus* strain 19 (S19) was introduced into the official Brucellosis Eradication Program for cattle in the 1940s [59]. Although the vaccine was an effective tool in the eradication program, there were challenges with the vaccine in that it interfered with diagnostic tests and was infectious for humans [59]. After many years of research, a new vaccine, *B. abortus* RB51 (RB51), was approved by the United States Department of Agriculture (USDA) and became commercially available in 1996. RB51 is now the official bovine brucellosis vaccine used in the USA [59].

In addition to strain 19 and RB51 for cattle, *B. melitensis* Rev. 1 (Rev. 1) vaccine is used for the prevention of brucellosis

in sheep and goats [60]. There are no brucellosis vaccines available for swine [60] at this time, nor are there brucellosis vaccines available for humans [61].

Brucella abortus Strain 19

S19 was first isolated from the milk of a Jersey cow in 1923. After the culture was maintained at room temperature in a laboratory for a year or more, it was found to have become attenuated [13]. In 1930, Buck reported the apparent lack of virulence and protection in a few heifers. After further studies on safety and efficacy, S19 was introduced for field usage in 1941 [53].

Since S19 is a smooth strain, it has the potential to induce antibody responses in most brucellosis serological tests that cannot be differentiated from that caused by infection with field-strain *B. abortus* [62]. A small percentage of animals will become persistently infected with S19 into adulthood, causing persistent titers that interfere with serological surveillance [63]. Brucellosis vaccine-induced antibody responses do not correlate with long-term protective immunity in animals [64]. High serological titers in S19 vaccinated animals correlate with chronic infection rather than resistance [65].

Vaccination with S19 does not induce full immunity, but it does increase resistance to *B. abortus* and has an efficacy estimated to be about 70–90% [61, 66]. However, because of the complications caused by residual antibody titers that interfere with surveillance and because S19 vaccination can cause abortions in some pregnant animals, vaccination was initially limited to calves, which received a full dose [$2.5–12 \times 10^{10}$ colony forming units (CFU)] of vaccine [57]. Following calfhood vaccination, S19 is usually cleared from superficial cervical lymph nodes within 10–12 weeks [57].

In the USA, due to difficulties in eliminating brucellosis from some herds and due to economic considerations, vaccination of adult cattle with S19 was explored. As a result, the Brucellosis Eradication Program was amended in 1977 to allow for the vaccination of adult cattle with a reduced dose of S19 [67]. A reduced dose administered subcutaneously to adult animals was an effective alternative that produced protection without a persistent antibody response and reduced the risks of abortion and excretion of vaccine-strain bacteria in milk [61].

Additionally, S19 is pathogenic to humans and thus requires the use of personal safety equipment such as gloves and protection glasses [57]. Since 1996, S19 vaccination has been replaced in the USA by RB51, primarily to eliminate persistent infection and titers that complicated diagnosis and surveillance efforts.

Brucella abortus Strain RB51

RB51 was first prepared commercially in 1996 and is now the brucellosis vaccine of choice for cattle in the USA [59]. RB51 is a rifampin-resistant rough mutant of virulent *B. abortus* strain 2308, derived by serial passages on media containing rifampin [60]. Since it is a rough mutant, RB51 is essentially devoid of the LPS 0-side chains on the bacterial surface [61]. These 0-side chains, that are present in field strains and S19, are the immunodominant antigens in the production of antibrucella antibodies. Since RB51 lacks this antigen, vaccination does not produce antibodies that react in the routine serologic tests for brucellosis, thus allowing the ability to distinguish vaccinated animals from those naturally infected [61] – an extremely important component of a disease eradication program. RB51 has been shown to induce a protective cell-mediated immune response against experimental challenge with the virulent strain 2308 [63].

Numerous studies have shown that RB51 offers significant protection from abortion, although there is no significate difference in effectiveness between RB51 and S19 [59]. In the USA, calfhood vaccination with RB51 is licensed for non-pregnant heifers 4–12 months of age. The vaccine must be administered by a state or federal animal health official or by an accredited veterinarian, and calves must be identified as vaccinated by an official eartag and a vaccination tattoo placed in the right ear [68].

Although cases are rare, RB51 can infect humans and is rifampin resistant. As a consequence, although rifampin is one of the drugs recommended for postexposure prophylaxis, it cannot be used for exposure to RB51 [69]. Therefore veterinarians should also use personal protective equipment such as gloves and glasses when vaccinating with RB51.

Brucella melitensis Rev. 1

Rev. 1 vaccine was developed to protect goats against *B. melitensis*, based on research conducted in Malta in 1956, and is the vaccine of choice for use in small ruminants [62]. Recommendations are to administer the vaccine subcutaneously in young animals (less than four months) and conjunctivally in older sheep and goats, avoiding those that are pregnant as the vaccine may induce abortions [64]. Since Rev. 1 is a smooth strain, it can induce serological titers that interfere with surveillance. Administering the vaccine conjunctivally reduces the likelihood of serological responses, without significantly reducing the protective effects of the vaccine [62].

Similar to other live *Brucella* vaccines, Rev. 1 is virulent for humans and precautions must be taken to avoid exposure [61]. At this time, since the USA is free of

brucellosis caused by *B. melitensis*, Rev.1 vaccine is not used in the country.

Human Infection

Brucellosis is a zoonotic disease, with a major impact on public health worldwide. Brucellosis in humans is also commonly known as undulant fever or Malta fever [70]. Annually, more than 500 000 human cases are reported worldwide, but it is suspected that human brucellosis is significantly underreported due to the non-specific clinical signs in humans and inadequate laboratory diagnosis in many countries [71]. Human brucellosis presents with a wide spectrum of clinical signs, thus complicating diagnostics as it often mimics many other diseases [72]. The disease is characterized by fever, anorexia, malaise, and profound muscular weakness [72]. In addition to those mostly non-specific symptoms, humans with brucellosis often suffer serious complications such as *Brucella* endocarditis, neurobrucellosis, or spondylitis. Chronic disease and relapses are frequent [71].

Although *Brucellae* are primarily host specific, four species of *Brucellae* are pathogenic for humans [8]. Those are *B. melitensis*, which causes the most severe disease in humans, *B. suis*, *B. abortus*, and *B. canis* [73]. Isolating and identifying the *Brucella* species involved in a human case can provide a clue as to the likely source of infection due to the limited number of preferred natural host animals [8].

There are several primary methods of transmission of brucellosis to humans. Studies have shown that a human's risk of contracting brucellosis are closely linked to food habits, standards of hygiene, and animal husbandry methods [74]. Human brucellosis is often the result of occupation-related exposure, primarily affecting adult men engaged in some aspect of the livestock industry [8]. This includes farmers, slaughterhouse workers, and laboratory workers who may be exposed through contact with infected animals or their products, or via aerosolized bacteria or entry of the bacteria through cuts and abrasions of the skin [73]. Veterinarians may also be infected by accidental inoculation with live *Brucella* vaccines [72]. Ingestion of contaminated dairy products is another very common method of transmission in much of the world and can affect a much broader segment of the population, primarily due to the consumption of fresh, raw milk and cheeses made from unpasteurized milk [72].

In the USA, human brucellosis cases decreased during the course of the brucellosis eradication program in livestock, with a dramatic drop in cases starting around the mid-1940s [20]. Currently, about 100 cases of human brucellosis per year are reported to the Centers for Disease Control and Prevention (CDC). Those cases are most often associated with international travel and the consumption of unpasteurized milk and dairy products from animals infected with *B. melitensis* [20]. Therefore if a case of human brucellosis is suspected in the USA, it is important to obtain a history of international travel and the consumption of dairy products abroad [8].

However, there is still a risk of *B. suis* infection to humans in the USA, primarily hunters and slaughterhouse workers, due to the prevalence of brucellosis in feral swine [30]. As the geographic spread of feral swine increases, there is an increasing threat of both recreational and occupational human exposure to swine brucellosis [75].

There have been three cases of human brucellosis caused by RB51 in the USA from the ingestion of raw milk from vaccinated cows [76]. Although very rare, with only three such cases identified in 2017 and 2018 despite extensive use of RB51 in the USA since 1997, these cases indicate there is a slight risk of acquiring vaccine-associated brucellosis from the consumption of raw milk.

Human brucellosis is routinely treated with long-term antibiotic therapy using a combination of agents [72]. Antibiotics are usually used in dual or triple combinations and may vary depending on the organ system involved and other factors. Antibiotics commonly used include doxycycline, streptomycin, rifampicin, and ciprofloxin [77].

Due to the risk of human infection, precautions should be taken when dealing with animals and animal products potentially infected with brucellosis. Standard personal protection such as gloves and protective clothing should be used, and personal hygiene such as carefully washing hands and other exposed areas of the body with soap and water should be utilized. The consumption of unpasteurized milk and dairy products should be avoided [73].

Future Research Directions

Brucellosis has been present in human and animal populations for centuries. Although there has been significant progress in the knowledge of the disease and the development of diagnostics and vaccines to control it, much work still remains to be done.

One of the challenges to future research is that *B. abortus*, *B. melitensis*, and *B. suis* could potentially be used as bioterrorism agents, and thus they are classified as "select agents" by the US Government [78]. "Select agents" are "biological agents that have the potential to pose a severe threat to animal health and safety, plant health and safety, or to the safety of animal or plant products (Select Agents)" [79]. The CDC and USDA Animal and Plant Health Inspection Service (APHIS) share regulatory

responsibility for some agents, including *Brucella* spp., because they potentially threaten both humans and animals [79]. Therefore any work with those pathogens must be registered with those agencies, accompanied by the appropriate permits, and closely regulated [78]. Most countries also require Biosafety Level 3 containment for animal facilities or laboratories conducting research with *Brucella*, thus limiting progress in research due to the expense of building and operating such facilities [80]. Striking an appropriate balance between the facilitation of *Brucella* science and national safety and security remains a challenge for the future of brucellosis research [80].

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Infectious Agents: Infectious Bovine Rhinotracheitis

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Introduction

Bovine herpesvirus-1 (BoHV-1), also known as infectious bovine rhinotracheitis virus (IBR), is a common pathogen of cattle responsible for significant economic losses due to respiratory and reproductive disease, pregnancy wastage, and the implementation of control measures. The virus affects cattle across the globe, although certain geographic areas have successfully eradicated the virus from local domestic cattle herds. BoHV-1 is recognized as an important pathogen in the bovine respiratory disease complex; clinical syndromes range from subclinical infection to severe disease. Infection also results in diseases of the reproductive tract, including abortion and genital syndromes. Due to the ubiquitous nature of the pathogen, its control in cattle populations is paramount to animal health and productivity.

Etiology

BoHV-1 is a virus of the Herpesviridae family and, as such, consists of a large, double-stranded, linear DNA genome. As a member of the subfamily Alphaherpesvirinae, BoHV-1 is characterized by a relatively short reproductive cycle and the tendency to establish latency primarily, but not exclusively, in the sensory ganglia. Three subtypes of BoHV-1 are recognized based on genetic analyses of clinical isolates [1]. Respiratory disease is generally caused by BoHV-1.1. The predominant strain in the USA, BoHV-1.1 may also cause abortions and infertility, often following infection of the respiratory tract. Genital infections are caused by BoHV-1.2. This subtype is further divided into BoHV-1.2a, which is abortifacient and occurs more frequently in South America, and BoHV-1.2b, which has not yet been associated with abortions and occurs in Australia, Europe, and the USA. The clinical syndromes associated with BoHV-1.2 infection are dependent on the route of transmission. Venereal transmission with BoHV-1.2 results in genital infections, whereas nasal infection can be followed by respiratory signs and transmission to other calves via the respiratory route. Therefore the manifestation of clinical signs is determined not only by the infecting viral strain, but also by the route of transmission. While differences have been noted in clinical syndromes caused by the different subtypes, genetic analysis remains the only definitive way to determine viral subtype.

The virus is sensitive to environmental inactivation. It is susceptible to ultraviolet light, desiccation, heat, and alterations in pH. Organic solvents and many disinfectants are effective against BoHV-1. The virus can survive the cryopreservation process for semen, and artificial insemination using contaminated semen can serve as a route of transmission in susceptible cattle [2].

Epidemiology

Disease associated with BoHV-1 infection is relatively common in almost all countries in which cattle are raised, with the current exception of some regions that have implemented strict control and/or eradication programs. Seroprevalence rates vary widely between countries, but may range from 10 to 70% depending on vaccination status, management practices, and the degree of contact with infected cattle. The virus is considered endemic throughout the USA. While seroprevalence rates may differ between beef and dairy herds within the same geographical area, these differences are more likely a reflection of different management programs than inherent breed differences [3].

Latently infected cattle serve as the reservoir for the disease, although a number of domestic and wild ruminants

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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may also harbor the virus. Among others, natural infection has been described in goats, wildebeest, pronghorn antelope, and a variety of cervid species. While sheep and camelids may become infected with the virus, they are unlikely to play a major role in viral transmission [4, 5].

Morbidity rates in unvaccinated feedlot cattle generally approach 20–30%; morbidity in dairy herds and cow-calf operations usually tend to be slightly lower. Occasionally, outbreaks with near 100% morbidity may occur depending on the viral strain and the immune status of the cattle. Uncomplicated infections with BoHV-1 are not usually associated with high mortality. Most deaths are due to secondary bacterial tracheitis and bronchopneumonia. The case fatality rate may reach 3–5% in an outbreak with a particularly virulent strain but more typically does not exceed 1%.

Pathophysiology

Direct nose-to-nose contact is the primary means of BoHV-1 transmission; aerosol transmission of the virus in nasal secretions and respiratory droplets over short distances has also been demonstrated. After infection through the respiratory mucous membranes and local replication of the virus, a primary viremia allows spread of the virus to the respiratory, reproductive, and ocular tracts; retrograde axonal spread allows further dissemination of the virus through the nervous system, even in the presence of humoral neutralizing antibodies. Viral infection and replication is not always prevented in young calves despite adequate colostral intake, which may result in latency and subsequent recrudescence after a decline in colostral immunity.

Infection resulting in the genital forms of the disease is acquired through venereal transmission and the genital carrier state is the primary reservoir for outbreaks of genital BoHV-1 disease. Venereal transmission may occur through natural service or artificial insemination. Virus shed in semen is due to viral replication in the mucosa of the prepuce, penis, and urethra rather than the gonads or accessory sex glands [2]. Genital shedding of BoHV-1 can be seen as early as two to seven days following infection and bulls may intermittently shed virus throughout life without showing clinical signs of infection. Infection is not believed to negatively impact sperm motility or semen quality [6] directly but may cause changes as a result of generalized illness.

Infection with BoHV-1 is characterized by viral latency. As with all Alphaherpesvirinae, latency occurs in the neurons, most commonly in the trigeminal ganglion in cases of BoHV-1.1 [1]. Latency also occurs in the germinal centers of the pharyngeal tonsils. Following venereal transmission, the site of BoHV-1.2 latency is usually the sacral ganglia. The virus may remain latent in the animal indefinitely or recrudesce following periods of stress such as transportation or the administration of exogenous corticosteroids. Latently infected animals do not consistently shed virus and do not always have detectable levels of neutralizing antibodies in serum. Therefore detection of infection in these animals is achieved by viral detection following administration of dexamethasone and recrudescence of the virus. Vaccination of animals with latent infections does not prevent shedding of the field strain already present. Due to latency, animals from endemic herds should always be considered as potential reservoirs of BoHV-1 and potential sources of infection to naïve herdmates.

Clinical Presentations

Abortion

In the USA and elsewhere, BoHV-1 is the most commonly diagnosed viral cause of bovine abortion. In Europe, BoHV-1-induced abortion is less commonly observed but could be underestimated [7]. Abortions due to BoHV-1 generally follow systemic (e.g. respiratory tract) infection, with subsequent spread of the virus from the site of primary infection to the fetus. Spread to the fetus is believed to occur hematogenously by peripheral leukocytes via the umbilical vein [8]. Fetal infection is generally fatal and the abortus is usually expelled within a few weeks of fetal death, although in some cases expulsion may be delayed for weeks or months; a delay in expulsion may result in fetal mummification. Abortions are most commonly seen in late gestation (five to eight months). Gross lesions are often absent from the aborted fetus, although placental lesions including blanched and degenerate cotyledons are common. Placental degeneration is considered to be secondary to fetal death rather than the instigating cause of the abortion. The virus may lie latent in the placenta for up to three months without transmission to the fetus; abortion may occur subsequent to activation of the virus and infection of the fetus.

Genital Forms

Genital infection with BoHV-1.2 causes infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP) in the cow and bull, respectively. Genital infections occur following venereal transmission of the virus. In both syndromes, clinical signs are usually observed one to three days following infection and include fever, anorexia, depression, restlessness, and frequent posturing to urinate. Inflammation of the vulva and vagina in cows, or of the preputial or penile surface in bulls, can be quite painful and result in hyperemia and the development of small raised pustules. The pustules may coalesce and form ulcers as the

disease progresses. Preputial prolapse can be observed in bulls and both sexes are susceptible to secondary bacterial infection leading to purulent discharge. Of particular concern in the bull, preputial adhesions may form as a result of the lesions and hinder future fertility. Treatment is generally not required and lesions usually resolve on their own within two weeks. Purulent vaginal discharge may persist in affected cows for several weeks. Likewise, libido in bulls may be decreased and resumption of normal breeding behavior delayed for several weeks [8].

Respiratory Disease

Infection with BoHV-1 results in inflammation of the upper respiratory tract, and hence the alternative name for the virus, IBR. Severity of clinical signs varies widely based on the strain virulence, host immunity, and environmental factors. Severe hyperemia of the nasal mucosa leads to the common moniker of "red nose." Other clinical signs include marked pyrexia, anorexia, ocular and nasal discharge, and focal necrosis of the mucous membranes of the nares. Necrotic foci may progress to large hemorrhagic and ulcerated areas covered by a diphtheritic membrane. Congestion and hemorrhage may be widespread throughout the trachea when visualized at necropsy or on endoscopic examination. Abnormal tracheal sounds can be readily auscultated and may not be accompanied by abnormal lung sounds unless there is secondary bacterial involvement that develops due to widespread inflammation and depression of innate immune functions of the upper respiratory tract. Development of bacterial bronchopneumonia is of higher consequence than viral infection alone as clinical disease following viral infection alone is often relatively mild, particularly in the USA. Secondary infection increases both the severity of clinical signs and overall mortality.

Other Clinical Syndromes

As with the other alphaherpesviruses, BoHV-1 has been implicated in clinical syndromes outside the reproductive and respiratory tracts. Conjunctivitis often occurs in conjunction with rhinotracheitis but may be the primary, or sole, clinical sign following viral infection. Conjunctivitis due to BoHV-1 may be unilateral or bilateral. In contrast to pinkeye, lesions are usually limited to the conjunctiva which is reddened and edematous; corneal ulcerations are not observed, although secondary corneal edema may be. Ocular discharge is common and is usually serous unless secondary bacterial pathogens are involved. Encephalitis in calves is occasionally associated with BoHV-1 infection but is more commonly seen with BoHV-5 (formerly BoHV-1.3) [9]. Systemic disease in newborn calves may be severe and characterized by high mortality. Calves less than 10–14 days of age may develop sudden anorexia, fever, rhinotracheitis, and conjunctivitis, particularly in herds with poor or diminished herd immunity and a lack of passive transfer of colostral antibodies.

Diagnosis

Virus Isolation or Detection

Isolation of the virus or detection of the viral genome using PCR in clinical samples can be used to demonstrate viral infection. Virus isolation has historically been considered the "gold standard" diagnostic test but has been largely replaced by PCR in many diagnostic laboratories due to the quick turnaround time of PCR analyses and the expense of cell culture programs required for virus isolation. The sensitivity of both virus isolation and PCR is relatively high [10]. Virus can be detected in a variety of samples including nasal, conjunctival, and vaginal swabs, preputial washings, bovine fetal serum, nasal secretions, and tissues samples, including those from an aborted fetus. Virus can also be detected by PCR in contaminated semen [11]. Other methods of viral detection include various immunofluorescence techniques, immunoperoxidase assays, and electron microscopy, although these are uncommonly utilized outside of research settings.

Serology

Evidence of BoHV-1 infection can be demonstrated by the detection of a serum antibody titer to the virus. Positive serology samples cannot differentiate between natural infection and vaccinal exposure using conventional vaccines such as those that predominate in the US market. Paired serology samples will help establish recent infection, as evidenced by at least a fourfold rise in titers between the acute and convalescent phases of infection. Antibody titers can be detected with either a virus neutralization test or enzyme-linked immunosorbent assay (ELISA). The primary immune response generally results in a detectable serologic titer by day seven post-infection.

Diagnosis of BoHV-1 Abortions

Diagnosis of bovine abortion can be challenging for the practitioner, as a definitive cause of abortion is not obtained in the majority of cases; the percentage of successful diagnoses increases when a full complement of diagnostic samples is submitted to the laboratory. For a complete

discussion of the diagnostic workup, the reader is referred to Chapter 56. Submission of the aborted fetus and the entire placenta provide the best opportunity for establishing a diagnosis of viral abortion. Admittedly, poor availability of either sample, particularly the placenta, may preclude submission, but the client should be advised of the benefit of locating and submitting both samples when possible. Diagnosis of BoHV-1 abortion is primarily based on histopathologic changes in the abortus and placenta. Moderate to severe autolysis of the fetus with focal hepatic necrosis may be present, but other gross changes are usually lacking. Intranuclear inclusion bodies may be visualized on tissue slides. Demonstration of viral antigen or genetic material in the abortus supports a diagnosis of viral abortion.

Prevention and Control

In the USA, vaccination is the primary means of controlling BoHV-1. However, the importance of designing and implementing an effective biosecurity program should not be overlooked, particularly relating to the introduction of new herd additions and the shipment of cattle that will eventually return to the home farm, as in the case of show cattle or certain breeding stock. Eradication programs have been successful in eliminating the disease from several European countries, and other countries within the European Union are in various stages of progressing toward complete abolition of the virus from domestic herds [12].

The goals of vaccination are to prevent clinical disease in the vaccinate as well as to protect fetal infection in animals that are, or will become, pregnant. Vaccination against BoHV-1 stimulates a strong antibody response in cattle. Both inactivated and modified-live virus (MLV) vaccines are widely available commercially, often in combination with other viral respiratory pathogens. Inactivated vaccines are often selected for their increased safety, while MLV vaccines are chosen for their rapid immune response, long-lasting immunity, and development of both local and mucosal immunity. Currently, parenteral MLV vaccines labeled for vaccination of pregnant cattle are available commercially; however, concerns have been raised that the vaccine may be associated with fetal loss following vaccination in isolated cases [13]. Consequently, one may consider pairing primary vaccination with an MLV vaccine and booster vaccination with inactivated vaccines in order to capture the benefits of both vaccine types while minimizing the risk of adverse side effects on the fetus [14]. Intranasal BoHV-1 vaccines are commercially available for use in both calves and adult cattle. They generally provide rapid local immunity and can be used in the face of an ongoing outbreak [15].

Serological assays cannot be used for the differentiation of infected from vaccinated animals (DIVA) when conventional vaccines are used. In Europe, genetically modified marker vaccines with a deletion in the glycoprotein gE gene are widely used as part of regional or federal control programs. Marker vaccines are DIVA-adherent in that serological detection of gE-specific antibodies denotes natural exposure since vaccinates will not have gE-specific antibodies. This principle also holds true for calves fed colostrum from dams vaccinated with the marker vaccine [16]. Marker BoHV-1 vaccines are currently not commercially available in the USA currently.

Summary

BoHV-1 remains a significant pathogen of cattle around the world, resulting in significant economic losses. Seropositivity rates are relatively high where eradication programs are not in place, although most infections do not result in severe clinical signs. The main manifestations of disease in the USA are abortion in breeding herds and as a component of the bovine respiratory disease complex in growing calves. Genital manifestations of IPV and IPB are more commonly reported in Europe. Less commonly, conjunctivitis, encephalitis, and systemic disease of neonates may result from infection.

Control of the disease is made difficult by the ability of the virus to become latent in the neural tissue and recrudesce following times of stress or immunosuppression. Strict eradication programs have eliminated the virus from some European countries; in the USA, vaccination and biosecurity are the core principles of most control programs. Vaccination is widely practiced and stimulates a strong antibody response. The use of MLV BoHV-1 vaccines in pregnant cattle should be balanced with an understanding of the potential risks to the fetus. Control of BoHV-1 is likely to remain a significant concern for cattle producers for the foreseeable future; an understanding of the vaccine types available coupled with knowledge of the management scheme of the individual producer will allow formation and implementation of the best control program for the virus.

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Infectious Agents: Bovine Viral Diarrhea

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Introduction

Bovine viral diarrhea virus (BVDV, now referred to as pestivirus A or B) is a pathogen of cattle and other artiodactyls with substantial impact on the health and well-being of affected livestock. Originally described by two North American research groups in 1946 [1, 2], infections with BVDV are still cause for enormous financial losses to the cattle industry worldwide. While the two earliest reports on BVDV described a disease that was mostly of severe acute nature, less severe subacute cases were also observed [1, 2]. In the decades following their original descriptions, BVDV infections in immunocompetent animals were reported to be mainly subclinical, and it was estimated that 70–90% of acutely infected animals did not display clinical signs [3]. The perception of BVDV as a mainly apathogenic bovine virus changed in the early to mid-1990s following the description of two new BVDV-associated syndromes, hemorrhagic syndrome and severe acute BVD, that were responsible for severe clinical signs with mortality rates of up to 100% [4, 5]. Genetic sequencing of BVDV isolates from severe outbreaks led to the description of a second genotype of BVDV (BVDV 2) based on its genetic dissimilarity of greater than 30% from classic BVDV strains [5, 6]. Subsequent retrospective genetic typing revealed that BVDV 2 had been present in Ontario as early as 1981 without causing severe disease symptoms [4]. Variation in the severity and observed clinical signs among BVDV-associated disease outbreaks emphasizes the influence of the tremendous genetic variability of BVDV isolates.

BVDV is an RNA virus and is the prototypic member of the genus *Pestivirus* within the family Flaviviridae. Recent revisions to the taxonomic classification of the genus *Pestivirus* added seven new species, and the genus now contains eleven recognized species: BVDV 1 (pestivirus A) and 2 (pestivirus B), classical swine fever virus (CSFV; pestivirus C), border disease virus (BDV; pestivirus D), pronghorn pestivirus (pestivirus E), Bungowannah virus (pestivirus F), giraffe pestivirus (pestivirus G), HoBi-like pestivirus (pestivirus H), Aydin-like pestivirus (pestivirus I), rat pestivirus (pestivirus J), and atypical porcine pestivirus (pestivirus K) [7]. The lack of strict host specificity and antigenic relatedness of pestiviruses has caused difficulties with the traditional methods of classification based on the host from which a strain was isolated, and more accurate taxonomic information is now based on monoclonal antibody binding assays and phylogenetic analysis of genomic sequences [8–11]. Although sequence variations are located throughout the entire genome, the 5′ untranslated region (UTR) is most commonly used for the differentiation of pestiviral species [8–10, 12].

Pestiviruses are enveloped viruses with a single-stranded RNA genome that is approximately 12.5 kb in length and contains a single large open reading frame flanked by UTRs at the 5′ and 3′ termini [13]. During replication of BVDV in the host cell cytoplasm, mutation and variation occurs readily, with an approximate mutation frequency of 10^{-4} base substitutions per base site [14]. At this high mutation frequency, at least one point mutation occurs per replication cycle of the viral RNA [15]. Frequent mutation is the consequence of error-prone viral RNA polymerases and results in variation of each progeny virus from the infecting parent virus [14]. Although millions of varying progeny virions are produced daily during the peak of infection, only copies with a selective advantage and without deleterious mutations will progress in the course of an infection [14, 16]. Consequently, a swarm of mutant virions exists in the host during a BVDV infection [14]. The virions of this mutant swarm are similar in base sequence and are referred to as a quasispecies [14]. The existence of BVDV as a quasispecies

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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with frequent mutation events offers a significant advantage to viral survivability, as viral mutants that are readily available are capable of adapting to adverse conditions, such as the host's immunity. With respect to virulence, adaptation to either lower or higher virulence may benefit viral survivability. While low virulence and host survival, as are common for host-adapted viruses, enable prolonged shedding, viral mutations that increase rates of replication and virulence allow for a burst of viral shedding [14, 17].

BVDV isolates can be classified as non-cytopathic or cytopathic, and this classification is based on the effects of an isolate on cultured cells. Cytopathic isolates cause cytoplasmic vacuolation and death of cultured cells, but infections with non-cytopathic isolates are inapparent [18, 19]. The categorization into biotype is not associated with the virulence of a BVDV isolate, and mild or severe disease can be observed with either biotype. The non-cytopathic biotypes are more prevalent in nature, and 60–90% of isolates from specimens at diagnostic laboratories are non-cytopathic [20, 21]. Only non-cytopathic BVDV strains can induce persistent infections, and these non-cytopathic strains are believed to be the most common source for cytopathic BVDV strains following homologous or heterologous recombination in the NS2–3 genomic region [14]. A third biotype of BVDV, the lymphocytopathic biotype, has been proposed and refers to BVDV isolates that have cytopathic effects only on cultured lymphoid but not epithelial cells [22]. Lymphocytopathic isolates are BVDV 2 strains that were associated with severe clinical disease [14].

Prevalence

Following the initial descriptions in North America, BVDV has been detected in cattle populations worldwide. The prevalence of seropositive animals depends largely on the management of cattle, including type of husbandry, use of vaccination protocols, addition of new herdmates, and presence of persistently infected (PI) animals [23, 24]. In North America, serosurveys demonstrated seropositive rates between 40 and 90% [25–27]. The individual seroprevalence rate among unvaccinated dairy heifers in Canada for BVDV 1 and BVDV 2 was 28.4 and 8.9%, respectively, and the herd-level prevalence was 53.4% for BVDV 1 and 19.7% for BVDV 2 [28]. In feedlot cattle, seroconversion to respiratory pathogens including *Pasteurella haemolytica*, *Mycoplasma* spp., and BVDV following arrival occurred in 40% of cattle [29, 30], but significant variation between groups is observed [30]. Similar seroprevalence rates have been detected in cattle herds worldwide, but the observed range varies widely among countries, reflecting global differences in cattle management and disease prevention practices.

In contrast to seroprevalence rates, the prevalence of PI cattle is considerably lower and is generally believed to be less than 1% of all cattle. PI cattle may be found in clusters within groups of cattle, elevating the prevalence within populations [31]. The prevalence of PI calves arriving at feedlots was demonstrated to be between 0.1 and 0.3%, and this rate is similar to the reported rate of 0.17% for beef cow-calf operations in the USA [30, 32–34].

Host Range

Pestiviral infections have been identified in various species of the mammalian order Artiodactyla, and lack of host specificity may play an important role in the pestiviral survival strategy [35, 36]. Among pestiviruses, BVDV has been the species most intensely studied as an infectious agent of heterologous hosts. Evidence of BVDV infection exists in over 50 species of cloven-hoofed animals (Artiodactyla) [35]. The implications of heterologous pestivirus infections are twofold. First, heterologous BVDV infections may hamper the success of control strategies, as are in progress for BVDV in cattle and CSFV in swine. Second, heterologous BVDV infections may threaten the health and well-being of other mammalian hosts, such as free-ranging and captive wildlife. The role of heterologous infections in the epidemiology of BVDV is incompletely understood, but evidence for interspecific transmission and BVDV-associated disease has been reported in various species.

BVDV in Small Ruminants

The close phylogenetic relatedness of small ruminants to cattle and associated greater possibility of permissive infections may promote a unique role of sheep and goats in the ecology of BVDV. Sheep and goats often have closer contact with cattle compared with other artiodactyls, especially in certain management conditions such as subsistence agriculture, alpine farming, or hobby farming. While one report did not identify a positive correlation between BVDV seropositivity of sheep flocks and presence of cattle [37], other studies have reported significantly greater seroprevalence rates in sheep on farms with cattle, especially where communal alpine pasturing of sheep, goats, and cattle was practiced [38].

Worldwide, infection of small ruminants with BVDV is common and many serological surveys have demonstrated antibodies against BVDV in small ruminants. Seroprevalence rates among evaluated flocks or herds varies widely, depending on associated risk factors. Due to cross-reactivity of BVDV antibodies with other pestiviruses, it is important to determine which pestiviral species caused the infection to accurately determine measures of prevention and control [39].

Various serosurveys that further evaluated the infecting pestiviral species by virus neutralization assays determined infections with BDV to be more common in small ruminants [40, 41], but several others demonstrated BVDV as the more frequent infecting viral species [37, 38, 42].

Infections with BVDV in domestic small ruminants result in clinical signs of border disease. In a recent study from China, 17.5% of goats with diarrhea, respiratory disease, or mucosal inflammation were positive for BVDV, indicating that infection and clinical disease are not uncommon in some small ruminant populations [43]. As in cattle, postnatal BVDV infections of non-pregnant small ruminants typically cause mild clinical signs, including pyrexia anorexia, tachycardia, pneumonia, and leukopenia [44, 45]. In contrast, infection of pregnant sheep and goats results in uteroplacental pathology, pregnancy loss by fetal resorption or abortion, and birth of mummified or stillborn fetuses [46]. BVDV and related pestiviruses are important causes of abortion and congenital disease of sheep and goats worldwide [47–50]. Fetal death and non-viability are common sequelae of transplacental BVDV infections; however, reports of viable PI offspring exist [51–54].

BVDV in Swine

The close antigenic relationship between ruminant pestiviruses and CSFV has important implications for diagnostic testing and control of CSFV. Currently, examination of blood samples for CSFV antibodies relies on a combination of enzyme-linked immunosorbent assay (ELISA) and virus neutralization techniques, and considerable efforts are necessary in establishing a CSFV-specific diagnosis [55]. During the CSFV outbreak in the Netherlands in 1997/1998, 26.5% of CSFV ELISA-positive samples were caused by the presence of antibodies to ruminant pestiviruses [55]. While BVDV seroprevalence rates in swine are typically lower than detected in other livestock [56–59], infection rates are greater in some production systems. A study from China identified BVDV in 26.8% of pigs exhibiting clinical signs of fever, diarrhea, abortion, or piglet mortality [60]. Risk factors associated with BVDV infection of swine include the presence of cattle on the same farm, high density of small ruminants near swine populations, vaccination with BVDV-contaminated vaccines, age of tested swine, and failure to follow biosecurity measures [57, 61–63]. Postnatal BVDV infections in non-pregnant swine rarely result in clinical signs [64, 65]. Experimental infection of eight-week-old pigs with either BVDV 1 or BVDV 2 did not result in clinical signs or increases in body temperature in any group, despite the use of a BVDV 2 isolate that was demonstrated to be virulent for cattle [65]. Experimental inoculation of pregnant pigs with BVDV does not consistently induce transplacental infection [66, 67]; however, reports of reproductive failure and congenital disease exist [68–71]. Similar to congenitally infected ruminants, piglets can be born PI with BVDV; however, seroconversion and clearance of infection after several weeks to months is also possible in some congenitally infected pigs and is termed "chronic infection" [68, 71].

BVDV in New and Old World Camelids

BVDV infections were identified through detection of antibodies as early as 1975 in Old World camelids (OWC) and 1983 in New World camelids (NWC) [72, 73]. While initial seroepidemiologic and experimental infection studies suggested that BVDV, while able to cause infections in NWC, did not pose a serious risk in these species, the first description of a PI alpaca following natural exposure of its dam [74] changed this perception. Several cases of PI alpacas have since been reported in North America and the UK [75–77], prompting prevention and control measures focused on improved biosecurity and detection of PI animals.

The BVDV antibody prevalence rates in NWC in European, North American, and South American studies is generally lower than those of other livestock species, with an individual prevalence of approximately 5% detected by most studies [78–85]. In contrast, most studies investigating BVDV infections in OWC detected much greater seroprevalence rates, suggesting widespread BVDV infection in camels [86–92].

Postnatal infection of camelids with BVDV following experimental inoculation did not result in clinical signs; however, viremia, nasal shedding, and alterations of the leukogram were documented [93–95]. In contrast, infection of pregnant OWC and NWC may result in reproductive disease including abortion, stillbirth, weak calves, early neonatal death, and neonatal hemorrhagic disease [74, 96]. In one study, BVDV was detected in approximately 15% of aborted camel fetuses [48], indicating that the virus may have a significant impact on camel production. Another possible outcome of BVDV infection in pregnant camelids is birth of congenitally infected offspring. Interestingly, like pigs, congenitally infected alpaca crias may be PI or chronically infected [97]. Congenitally infected crias are affected by low birthweight, failure to thrive, inappetence, lethargy, chronic diarrhea, and chronic recurrent infections especially of the respiratory tract [74, 76, 97, 98]. As in other species, BVDV is shed readily by PI camelids, and shedding of BVDV in semen from a camel was also documented [99]. Therefore sound biosecurity practices, such as preventing movement of animals between farms for breeding purposes, are important to prevent intraspecific transmission of BVDV.

BVDV in Wildlife

Serologic evidence for BVDV infection exists in many species of free-ranging and captive wildlife, encompassing at least 7 of the 10 families in the mammalian order Artiodactyla, including Antilocarpidae (pronghorn antelope) [100], Bovidae (e.g. European and American bison) [101, 102], Camelidae (e.g. vicuña) [83], Cervidae (e.g. mule deer) [103], Giraffidae (giraffe) [104], Suidae (e.g. Eurasian wild boar) [105], and Tragulidae (e.g. lesser Malaysian mousedeer) [106]. Additionally, BVDV antibodies were demonstrated in a few non-artiodacyl hosts, including in the European hare and European rabbit [107, 108]. While these studies demonstrate widespread exposure of wildlife to BVDV, they typically cannot provide information about the source and importance of the detected BVDV infection. Contact with infected cattle is a likely source for BVDV and greater BVDV rates were demonstrated in some wildlife populations that have contact with cattle [109, 110]. Transmission of BVDV from cattle to white-tailed deer was demonstrated in a cohabitation experiment [111]. In that study, BVDV was efficiently transmitted from PI cattle to white-tailed deer and resulted in seroconversion in all adult deer and birth of PI fawns from four of seven pregnant does [111]. In contrast, independent circulation of BVDV not related to contact with cattle has also been demonstrated [112, 113] and is further suggested by high seroprevalence rates identified in some wildlife populations [103, 114–116]. Maintenance of BVDV within a cervid population without presence of cattle was suggested by the presence of antibodies in over 60% of caribou that had not been in contact with domestic ruminants for over 25 years [117].

Independent circulation of BVDV in wildlife would likely depend on the presence of PI animals, which have been described in different wildlife species, including common eland (*Taurotragus oryx*), lesser Malayan mousedeer (*Tragulus javanicus*), mountain goat (*Oreamnos americanus*), mule deer (*Odocoileus homionus)*, and white-tailed deer (*Odocoileus virginianus*) [106, 115, 118–120]. While shedding of BVDV by acutely infected and PI wildlife has been demonstrated [120–124], only a few studies have examined the transmission potential of infected wildlife to cattle or conspecifics [121, 123, 125–128].

Few reports on the clinical outcome of BVDV infection in wildlife exist, but reported clinical signs are similar to BVDV infections in cattle. Experimental inoculation with BVDV NY-1 did not result in clinical signs in four mule deer and one white-tailed deer, despite evidence of viremia and nasal shedding [124]. Similar findings were made in yearling elk that were inoculated with either BVDV 1 Singer or BVDV 2 24515 [123]. In two young reindeer, loose, bloody, and mucoid feces, transient laminitis, or coronitis were observed after inoculation with BVDV Singer and mild lesions were detected at necropsy [129]. Naïve white-tailed deer fawns developed moderate pyrexia and marked to moderate decreases in lymphocyte populations in response to either BVDV 1 or BVDV 2; and lethargy or coughing was observed in individual fawns [130, 131]. Similar to other species, reproductive failure may be the most important consequence of BVDV infections in wildlife. Depending on the stage of gestation, infection of pregnant white-tailed deer with BVDV may result in fetal mummification, stillbirth, and abortion [120, 132]. Currently, insufficient data exist to determine whether BVDV infections negatively affect populations of free-ranging wildlife, as has occurred with BDV, a closely related pestivirus which has decimated some populations of Pyrenean chamois (Rupicapra pyrenaica pyrenaica) populations in Spain, France, and Andorra [133–135].

Transmission

Several direct and indirect routes of transmission have been described for BVDV within and between cattle herds [136]; however, PI animals are considered the principal reservoir of the virus and the main source of BVDV transmission in cattle populations [137]. Direct contact of susceptible cattle with PI animals results in higher BVDV transmission rates compared with direct contact with acutely infected animals [136]. Lower amounts and shorter duration of viral shedding have been described in cattle acutely infected with BVDV compared with PI cattle [138, 139]. Considerable virus concentrations are present in oculonasal secretions, saliva, feces, urine, uterine discharges, semen, and milk of PI cattle [23, 136, 140]. Therefore continuous shedding of BVDV in secretions from PI cattle results in increased transmission rates to susceptible animals [136, 137].

Transmission of BVDV is highly efficient when PI animals are born or introduced into the herd [137]. In one study, all susceptible cattle that came in contact with a single PI animal became seropositive in less than three months [141]. The purchase of a PI animal or of a pregnant heifer carrying a PI fetus represents the greatest risk of introduction of BVDV into susceptible herds [137, 142, 143]. Purchasing pregnant heifers from facilities or operations that commingle many cattle from different origins facilitates the maintenance and dispersal of BVDV within cattle populations [142, 143]. The risk of introducing BVDV into a naïve herd through the purchase of a PI animal (including a PI fetus from a pregnant heifer) when buying cattle of unknown origin and without testing is estimated between 33 and 47% [137, 142]. Introduction of BVDV to naïve herds through the purchase of acutely infected cattle can also result in BVDV transmission. In one study, the risk

of introduction of an acutely infected animal when buying 20 cattle of unknown origin or testing status was estimated to be 8% [136, 137]. However, factors such as virulence of the BVDV strain and severity of clinical signs may affect the dynamics of viral spread and transmission [137].

While PI animals are the major source of BVDV, transmission in the absence of PI animals has been described [141, 144], indicating that other transmission routes are important in the epidemiology of the disease. The birth of non-surviving PI fetuses, fence-to-fence contact with infected cattle, contact with infected heterologous hosts, use of contaminated semen or vaccines, use of contaminated fomites, airborne transmission, and hematophagous insects have been demonstrated to be alternative routes of BVDV transmission [137, 144–148]. Transfer of embryos from or to PI or acutely infected cattle is an effective route of introduction of BVDV into susceptible cattle herds [136]. Use of contaminated fetal calf serum for embryo washing procedures is also an effective route of BVDV transmission through embryo transfer. Although it has been suggested that appropriate embryo washing eliminates the risk of BVDV contamination, one study demonstrated that recommended washing procedures were ineffective for removal of BVDV from an *in vitro* fertilization system [137, 149].

Clinical Disease Syndromes

Infections with BVDV may result in a wide variety of clinical manifestations as the outcome of infection depends on the triad of host-associated factors, environmental stressors, and viral characteristics [150]. Host-associated factors include (i) immune status and immunocompetence, (ii) pregnancy status, and (iii) gestational age of the fetus at the time of infection. Viral characteristics that influence the outcome of an infection include the biotype of the infecting strain and genotypic variation that determine virulence and antigenicity. Infections with both species of BVDV may result in a broad spectrum of clinical manifestations, but severe acute BVD, thrombocytopenia, and hemorrhagic syndrome, as reported in North America, were associated only with BVDV 2 [4, 5, 151].

Postnatal Infections in Immunocompetent Cattle

Postnatal infections in cattle that are able to immunologically respond to the BVDV infection are referred to as "acute" or "transient" infections and the severity of clinical signs depends on the infecting BVDV strain. After infection of susceptible cattle, the tonsils and respiratory tract are the first sites of BVDV replication. Subsequently, the pathogen disseminates to other epithelial and lymphoid tissues, resulting in infection of gastrointestinal, integumentary, and respiratory tissues [152, 153].

The majority of BVDV infections in immunocompetent seronegative cattle proceed subclinically [3]. However, close observation of infected animals usually reveals mild signs including hyperthermia, leukopenia, and decreased milk production. Affected animals develop neutralizing antibodies [154]. Symptomatic infections are most commonly observed in 6- to 24-month-old cattle following waning of maternal immunity, in colostrum-deprived calves, or in seropositive cattle as a result of infection with a heterologous BVDV strain [155]. Clinical signs in affected animals include pyrexia, lethargy, leukopenia, ocular and nasal discharge, oral erosions and ulcers, blunting of oral papillae, diarrhea, and decreased milk production. More virulent BVDV strains can also cause epithelial erosions at the interdigital spaces, coronary bands, teats, or vulva. Although BVDV-associated immunosuppression can result in pneumonia, the tachypnea observed in acutely infected animals is likely the result of hyperthermia and other nonpulmonary factors [155].

In the 1990s, severe acute BVD was described in cattle herds in the USA and Canada [5, 151]. The outbreaks were characterized by a peracute course and caused unusually high rates of morbidity and mortality in all ages of cattle. The described clinical signs and postmortem findings were principally those of mucosal disease, with severe diarrhea, fever, ulcers, and erosions of the upper alimentary tract [4]. Histopathologic findings included a dramatic lymphocytolysis and lymphoid depletion of Peyer's patches, necrosis of intestinal crypt epithelium, and diffuse ulcerative lesions in the upper alimentary tract. Sudden death without premonitory clinical signs, abortions, and pneumonia were also prominent findings in some affected herds. Interestingly, the gross and histopathologic postmortem changes were more pronounced and more likely to be observed in older animals, which is in contrast to most previous descriptions of transient BVDV infections [4]. The viral isolates from all reports of severe acute BVD were determined to be different from traditional BVDV isolates in genotype and monoclonal antibody binding patterns, prompting the designation BVDV 2 [5, 10, 156].

Another form of severe acute BVD associated with BVDV 2 is the hemorrhagic syndrome, which is characterized by marked thrombocytopenia. The hemorrhagic syndrome is observed only in a minority of cattle with severe acute BVD, contributing to a diverse clinical picture in an outbreak [155]. Clinical signs in affected cattle include petechiation and ecchymoses of mucosal surfaces, epistaxis, bloody diarrhea, bleeding from injection sites, trauma, fever, and death [151]. The marked thrombocytopenia and leukopenia of affected animals is accompanied by altered

function of platelets, and thus quantitative and qualitative platelet defects contribute to the observed hemorrhagic diathesis [157].

In addition to direct effects on the host organism, BVDV suppresses the number and function of various innate and adaptive immune components, further damaging tissues by enabling infections with secondary pathogens. BVDV-induced immunosuppression is especially important in polymicrobial diseases such as the bovine respiratory disease complex of feedlot cattle and dairy calves. The synergistic action of BVDV and BHV-1, BRSV, *Mycoplasma bovis*, and *Mannheimia haemolytica* in inducing severe respiratory disease in cattle has been previously documented [158–160]. Infection of cells of the innate immune system may result in impairment of function and decreases in the number of circulating leukocytes. The microbicidal, chemotactic, and antibody-dependent cell-mediated cytotoxicity was impaired in neutrophils that were infected with BVDV [161]. Infected monocytes may undergo apoptosis, and a reduction of 30–70% in the number of monocytes has been observed after BVDV inoculation of calves [162–164]. Infection with BVDV diminishes the ability of antigen-presenting cells to present antigens to T-helper cells by reduction of Fc and C3 receptor expression and downregulation of major histocompatibility complex (MHC)II and B7 molecules [162, 165–167]. A strain-dependent inhibition of function with reduced ability to kill bacterial and fungal pathogens was observed following BVDV infection of cultured macrophages; however, significant functional inhibition was induced only by virulent strains of BVDV [166].

Similar to innate immunity, adaptive immune responses are affected quantitatively and qualitatively by BVDV infections. Upon infection with BVDV, circulating lymphocytes are reduced and lymphocytes in lymphoid tissues are depleted, and this depletion is strain-dependent [162, 168]. Of the T-lymphocyte subpopulations, CD8+ cytotoxic T-lymphocytes are most dramatically depleted, followed by CD4+ T-helper cells, with little reduction of circulating γ/δ T-cells [169]. In addition to lymphocyte depletion, functional alterations of $CD4^+$ cells are pronounced, and non-cytopathic BVDV infections result in a shift toward the Th2 response, with reduced cell-mediated but high levels of humoral immunity [170–172]. Virulent BVDV isolates can cause reductions in MHCI expression of infected cells, thus affecting cytotoxic $CD8^+$ cell function; however, this reduction appears to be strain-dependent [166].

The humoral immunity to BVDV infection depends largely on the quantity of neutralizing antibodies and the antigenic relatedness of the infecting strain with that of the original exposure. Although an amount of cross-reactive protection is conferred by vaccination against heterologous strains, and even between BVDV 1 and 2, superior protection from infection appears to result from homologous exposure [173, 174].

Infections of the Reproductive Organs

While BVDV-associated immunosuppression and respiratory disease are causes for the greatest losses in feedlot and stocker operations, susceptible cow-calf and dairy herds suffer considerable economic damage from BVDV-induced reproductive failure. Reproductive losses as a result of BVDV infection can occur throughout gestation, and BVDV infection at the time of breeding can result in reduced conception rates and embryonic losses. In heifers experimentally infected with BVDV nine days prior to insemination, a conception rate of 44% was detected compared with 79% in control animals [175]. In the same study, other heifers were infected four days after artificial insemination (AI), resulting in a conception rate of 60% by day 20; however, significant embryonic loss was detected by day 79 when only 33% of animals were still pregnant. Detrimental effects of BVDV infection on fertility were also reported in field studies, in which poor conception rates, early embryonic losses, and pregnancy losses were detected [176–179]. In contrast, heifers exposed to PI calves for 220 days beginning 50 days prior to the breeding season had the same pregnancy rates as unchallenged control heifers, due to development of protective immune responses prior to breeding [180]. Highlighting the benefits of a controlled breeding season, this study demonstrated that BVDV infections can be self-limiting in certain herds, when all animals are protected from BVDV during gestation and PI calves are not born [180].

Both male and female gonads and the entire female reproductive system are permissible for BVDV replication [181]. In postpubertal heifers experimentally infected with a non-cytopathic BVDV, the virus was isolated from the corpora lutea of two animals, and BVDV-antigen staining was detected predominantly in the stroma of ovarian cortex. In that study, prolonged antigen staining and associated chronic lymphocytic oophoritis were detected from 6 to 60 days following intranasal inoculation [182]. Similarly, experimental inoculation of susceptible heifers with cytopathic BVDV resulted in chronic oophoritis for days 5–61 after infection, and BVDV was detected in the ovaries of a subset of cattle immunized with a commercial vaccine containing cytopathic BVDV [182, 183]. Ovarian inflammation may alter ovarian cytokine concentrations or disrupt tissue macrophages, thus interfering with normal ovarian dynamics, and while the effects of BVDV on ovarian function remain incompletely understood, alteration of follicular formation and inhibition of ovulation were demonstrated following BVDV infection [182]. A decrease in the number of subordinate follicles and retarded growth of dominant anovulatory and ovulatory follicles were observed in heifers experimentally infected with BVDV, but the infection did not result in alterations of peripheral estradiol or progesterone patterns [182]. In contrast, experimental infection with

non-cytopathic BVDV two days prior to estrus resulted in significantly reduced plasma estradiol concentrations but did not affect progesterone secretion or plasma prostaglandin F2α concentrations [181]. While the average number of follicles in each size-category was not affected in BVDV-infected superovulated heifers, infection resulted in the absence or delay of the preovulatory surge of luteinizing hormone, reduced number of corpora lutea and recovered ova/embryos, and oophoritis with necrosis of granulosa cells [184–186]. Alteration of sex hormone concentrations with potential for disruption of ovulatory follicles to form a competent corpus luteum was also detected in heifers experimentally inoculated nine days before synchronized ovulation [187]. Collectively, these studies suggest that transient BVDV infections can interfere with normal ovarian dynamics and result in transiently or prolonged reduced reproductive ability [186, 188]. Other causes for reproductive losses associated with BVDV infection at the time of breeding have been proposed and include disruption of the hypothalamic–pituitary–gonadal axis, deficiency of gonadal leukocytes, and incompatibility of the oviductal and uterine environment for embryonic development [181, 188]. Infection with BVDV was demonstrated in oviductal cells following intravenous inoculation with non-cytopathic BVDV and intrauterine infection with cytopathic BVDV resulting in salpingitis for 21 days [189, 190]. In uterine flushes from a subset of experimentally infected heifers, BVDV was isolated on days 8 and 16 following infection with BVDV, demonstrating a potential risk for the developing embryo in the uterine environment [190].

Widespread BVDV infection of the reproductive system and reduced reproductive performance are described in PI cattle. Extensive BVDV immunostaining and pathoanatomical abnormalities including significantly reduced follicular structures and ovarian and follicular hypoplasia were described in PI cows [191]. In addition to ovarian structures, including follicular epithelium, theca cells, cumulus oophorus, stromal macrophages, and walls of small ateries, BVDV antigen is present in oviductal epithelial cells, uterine glandular, and luminal epithelia, arterial walls, and smooth muscle of PI cattle [192, 193]. While oocytes in primordial and secondary follicles did not contain BVDV antigen in one study, approximately 20% of oocytes in all stages of follicular development contained BVDV antigen in another report [181, 193].

Infections of the Conceptus

Infections with BVDV can have deleterious effects on the pregnancy, and infections before the third trimester are most harmful. BVDV is highly efficient in crossing the placental barrier, and fetal infections are common with either biotype [138]. Increasing rates of fetal infection of 33, 33, 86, and 100% were detected in cows experimentally infected at 4, 11, 18, and 30–34 days post-insemination, respectively [194]. In ewes infected with BVDV on day 18 of gestation, the virus was detected by reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) in fetal tissues approximately 100 hours following infection, and BVDV-positive cells were more numerous in fetal than maternal tissues [195]. Fetal infections occur in an orchestrated fashion, beginning with infection of fetal membranes by 72 hours, followed by infection of bulbis cordis and fetal aorta by 7 days, and subsequent spread to many fetal tissues by day 10–14 [195, 196]. Further dissemination through fetal tissues can be observed as the infection proceeds, and a primary target organ of fetal BVDV infection is the brain [197]. While viral antigen can be detected in most tissues from PI fetuses, histopathologic changes are primarily detected in the brain, liver, and spleen [198]. Invasion of the fetal brain may occur in a "Trojan horse" mode of invasion, in which BVDV enters the central nervous system (CNS) within infected microglial precursors [199].

To date, the mechanism of BVDV entry into the fetus is poorly understood. Infection of pregnant cows with non-cytopathic BVDV results in pronounced upregulation of interferon (IFN)-stimulated genes such as ISG15 in the first week after infection, and blood concentrations return to baseline by 45 days post-infection [200, 201]. In heifers carrying PI or transiently infected fetuses, BVDV infection results in measurable serum IFN-I concentrations, but this antiviral activity is not capable of preventing fetal infection [201]. This may be due to failure of maternal IFN-I and antibodies to enter the fetal bloodstream as a result of the blood–placental barrier [201]. Likewise, BVDV infection results in measurable IFN responses in the fetal blood, which is pronounced in fetuses infected after the susceptible window for development of persistent infection [202]. While IFN responses are also present in PI fetuses and resulting PI calves, these responses are moderate and unable to eliminate the infecting BVDV in the absence of specific adaptive immune responses [202, 203].

The outcome of fetal infection depends on the viral virulence and biotype, and the gestational age. Fetal death and abortions can occur at any gestational age, but are most common during the first trimester [188]. Fetal death during early pregnancy may result in fetal resorption or mummification (Figure 62.1) and expulsion of the fetus may occur up to 50 days following exposure to BVDV [204]. Of four heifers infected between 29 and 41 days of pregnancy, two abortions and two fetal resorptions were observed [205]. While infections in the preimplantation phase (before day 40 of pregnancy) result in large numbers of pregnancy losses, fetuses that survive infection with non-cytopathic BVDV of low to moderated virulence from approximately



Figure 62.1 (a) Mummification of a bovine fetus following exposure of a pregnant cow to cattle persistently infected with BVDV between 70 and 100 days of gestation. *Source:* Courtesy Dr. Paul Walz, Auburn University. (b) Similar appearance of a mummified white-tailed deer fetus, as found in birthing nest. Fetal death was estimated to have occurred at 94 days of gestation, approximately 50 days after BVDV infection of the dam. The mummified fetus was born together with a viable persistently infected fawn after completion of the full gestational period. (c) Fetal membranes removed.

40 to 125 days become PI [206, 207]. Persistent infections can only occur with non-cytopathic BVDV strains [207, 208]. Experimental inoculation of seronegative cattle with non-cytopathic BVDV at 75 days of gestation results in persistent infection in up to 100% of fetuses, and this gestational age is commonly used to induce persistent infection in research studies [201, 203, 209, 210]. The pathophysiology of persistent infection is incompletely understood, but this phenomenon occurs in fetuses before completion of immune system development, when viral antigens are recognized as self antigens, resulting in negative selection of BVDV-specific lymphocytes [188]. The negative selection of reacting $CD4^+$ lymphocytes is highly specific and the exchange of a single amino acid in the BVDV antigen may be sufficient to stimulate immune responses in $CD4^+$ lymphocytes from PI cattle [211]. The lack of immune responses results in widespread distribution of BVDV throughout the host's tissues, and PI cattle shed BVDV in multiple secretions and excretions, including nasal discharges, urine, semen, colostrum, milk, and feces [212].

Fetal infections during the period of organogenesis (approximately 100–150 days of gestation) may result in congenital malformations usually associated with abnormal development of nervous tissues. Defects of the CNS include cerebellar hypoplasia (Figure 62.2), hydranencephaly, hydrocephalus, porencephaly, microencephaly, spinal cord hypoplasia, and a variety of ocular changes such as cataracts, optic neuritis, retinal degeneration, and microphthalmia [188, 213, 214]. Less commonly, congenital BVDV infection can cause CNS hypomyelination, which has been reported with both species of BVDV [215, 216]. In addition to CNS abnormalities, congenital BVDV infections can

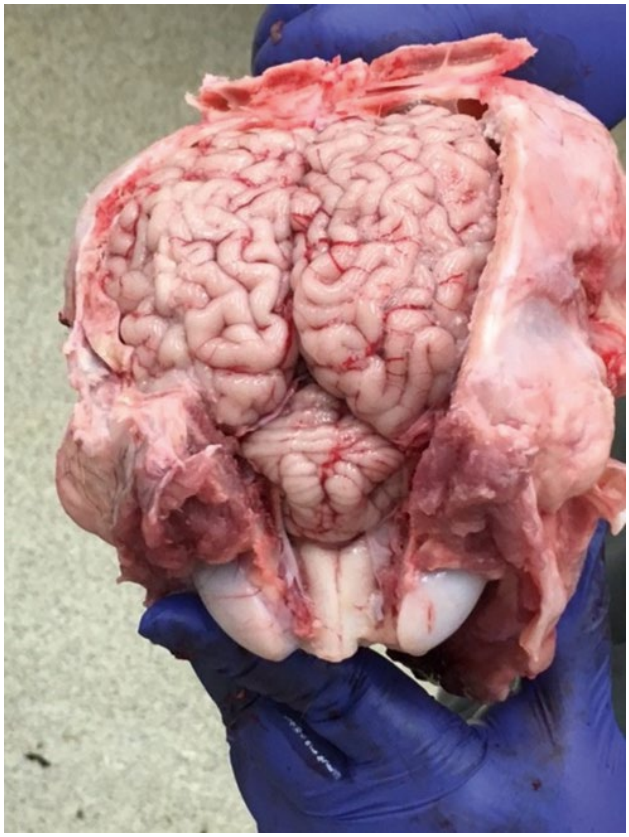


Figure 62.2 Cerebellar hypoplasia in a two-day-old Holstein calf. The calf had clinical signs of cerebellar disease including wide-base stance, intension tremors of the head, and bilateral horizontal nystagmus.

result in brachygnathism, osteopetrosis, thymic aplasia, hypotrichosis, hair coat abnormalities, pulmonary hypoplasia, and growth retardation [188, 212, 213, 217].

While abortions are still possible in later stages of gestation, congenital infections following the completion of organogenesis and immunocompetence (>150 days of gestation) result in effective immune responses and clearance of the virus. These calves are born with precolostral serum antibody titers and usually appear normal at birth [188]. However, one study suggested that calves born with precolostral antibodies against BVDV are more likely to develop severe illness during the first months of life, indicating that late gestational infections with BVDV may not be benign [218].

Infections in Persistently Infected Animals (Mucosal Disease)

Although PI calves may survive to reproductive age, many are born weak and stunted, and die shortly after birth [150]. PI cattle have a 50% greater risk of leaving the herd before the first year of age, dying, or being slaughtered due to unthriftiness as compared to normal cohorts, which may be partly due to a poor immune response and susceptibility to opportunistic infections [219, 220]. An additional risk for PI cattle is the development of fatal mucosal disease, which develops when cattle that are PI with a non-cytopathic BVDV become superinfected with a cytopathic strain. The severity of the developing clinical signs depends on the homology between both strains, and mucosal disease can be classified as acute, chronic, or delayed onset [221].

Acute mucosal disease occurs when the superinfecting cytopathic strain has close genetic homology with the PI strain, which most commonly is the result of mutational events that alter the PI strain's biotype [222]. Because of the close homology of both strains, acute mucosal disease results in case fatality rates that approach 100% [155]. Because acute mucosal disease affects only PI cattle, the disease is characterized by a low morbidity but very high mortality [155]. However, in groups of cattle with multiple PI animals infected with similar BVDV strains, outbreaks of mucosal disease have been reported [223]. Clinical signs of acute mucosal disease include fever, anorexia, tachycardia, polypnea, nasal discharges, and profuse foul-smelling diarrhea with mucosal shreds, fibrin casts, and blood. In addition, erosion and ulcers are commonly present on the alimentary epithelia (Figure 62.3), coronary bands, prepuce, and vulva [155].

Superinfections with heterologous cytopathic strains may result in chronic forms of mucosal disease that are not fatal but result in chronic clinical signs. The source for heterologous cytopathic strains is commonly vaccine-strains in modified-live vaccines [221]. Clinical signs include anorexia, weight loss, diarrhea, bloat, lameness, and erosive and ulcerative lesions on epithelial surfaces [221]. Sufficient heterology between the PI strain and the cytopathic strain may result in an immune response that clears the superinfection, enabling the animal's recovery from mucosal disease but not persistent infection [221]. Under experimental conditions, the incubation period of acute fatal mucosal disease is 7–14 days [224].

In contrast, delayed-onset mucosal disease occurs in PI cattle several weeks to months after inoculation with the cytopathic strain [225]. Similar to chronic mucosal disease, delayed-onset mucosal disease is caused by a heterologous cytopathic strain from an external source, but clinical signs occur after RNA recombination events between the PI strain and the heterologous cytopathic strain. This RNA recombination creates a homologous cytopathic strain and fatal mucosal disease [226].

Infections of Bulls

Both persistently and acutely infected bulls shed BVDV in semen, but the duration and level of shedding is much greater



Figure 62.3 Severe esophageal erosions and ulcers in a steer with acute mucosal disease. *Source:* Image courtesy of Dr. Jenna Bayne, Auburn, Alabama.

in PI bulls. In experimentally infected bulls, viral titers in semen (expressed in cell culture infectious dose [$CCID_{50}$]) ranged from 5 to 75 $CCID_{50}$/ml, and virus isolation was similarly effective from raw and extended semen [227]. In a group of heifers experimentally inseminated with contaminated semen from an acutely infected bull, approximately 5% became systemically infected [228]. While this initial rate of infection was low, it was sufficient for further transmission to susceptible heifers and maintenance of BVDV in the herd [228]. Under field conditions, AI with semen from two acutely infected bulls resulted in infection rates in exposed cows of 15.4 and 1.8%, respectively [229].

Semen from PI bulls can contain large quantities of BVDV (10^3–10^7 $CCID_{50}$/ml) [230, 231]. Insemination of susceptible heifers with semen from PI bulls consistently results in infection [230–232]. Semen quality from PI bulls ranges from normal on the basis of concentration, motility, and morphology of spermatozoa to abnormal, with morphological abnormalities of spermatozoa and low concentration and motility [230, 233].The use of semen from PI bulls usually affects conception rates negatively, which is likely due to a combination of factors, including lower quality semen, ill-thrift of affected bulls, and effects of BVDV on the reproductive tract of exposed cows [188, 230, 234].

In acutely infected bulls, formation of neutralizing antibodies results in viral clearance from most organ systems, but chronic infections of testicular tissues despite formation of serum antibodies are described [235]. This phenomenon was first described in a seropositive non-viremic bull that consistently shed BVDV in semen, suggesting an acute BVDV infection prior to completion of a functional blood–testes barrier [236]. Insemination of seronegative heifers with the bull's semen containing less than 2×10^3 $CCID_{50}$/ml of BVDV resulted in infection and seroconversion [146]. Localized and prolonged testicular infections have also been experimentally produced following intranasal inoculation and subcutaneous vaccination of peripubertal bulls with BVDV [237, 238]. Viral RNA was detected in semen for 2.75 years following BVDV exposure, and infectious BVDV was isolated from testicular tissue for up to 12.5 months after BVDV exposure [239]. Semen from a bull with prolonged testicular infection resulted in BVDV transmission when given intravenously to a seronegative calf [237]. However, artificial or natural insemination did not result in transmission of BVDV, indicating that the chronic presence of BVDV in testicular tissues could be of limited epidemiologic importance as a result of neutralization of infectious virus by humoral immunity in the ejaculate [235, 239].

BVDV and *in vivo* and *in vitro* Derived Embryos

Potential introduction of BVDV to *in vivo* and *in vitro* derived embryo production systems can result from three sources – donor cows, recipient animals, and materials of animal origin such as fetal bovine serum – and all three have to be free from BVDV to prevent transmission [240]. The World Organisation for Animal Health (OIE) states that "BVDV is a particularly important hazard to embryo transfer and the manufacture of biological products for other diseases due to the high frequency of contamination of batches of fetal calf serum" [241]. *in vivo* derived embryos could be exposed to BVDV if they were collected from acutely infected or PI cattle, or if contaminated sera were used in embryo collection, culture, or cryopreservation media [240]. However, BVDV appears to present a more serious threat in the production of embryos via *in vitro* production than via *in vivo* derived embryos. The virus can be introduced via serum or abattoir-origin oocytes and somatic cells used in *in vitro* embryo production. Hence unrecognized infections with non-cytopathic isolates could lead to amplification of the virus throughout *in vitro* maturation of oocytes, *in vitro* fertilization, and *in vitro* culture of embryos [242]. Furthermore, the virus

readily adheres to the zona pellucida of *in vitro* produced embryos, rendering the embryo washing and trypsin treatment procedures even less effective than they are for *in vivo;* derived embryos [243]. Production of embryos by somatic cell nuclear transfer presents even more unique hazards than that of *in vivo;* derived or *in vitro* produced embryos. Introduction of BVDV via serum used to culture both cytoplast-donor cell lines and the resulting embryos can readily occur. The cultured embryos are especially vulnerable because the zona pellucida has been either broken or removed to facilitate the process of cell fusion. Moreover, cumulus–oocyte complexes are usually collected at abattoirs from donors of unknown BVDV status and cell lines are often of fetal origin, creating enhanced opportunities for introduction and amplification of BVDV as embryos are produced and develop [242]. Therefore BVDV still continues to be a concern in embryo technologies and a more detailed discussion on this topic is included in Chapter 86.

Diagnosis, Prevention, and Control

As for other infectious pathogens, the main principles of BVDV control are the elimination of pathogen reservoirs and the prevention of transmission from infected to susceptible animals. Any BVDV control program should focus on three major principles: (i) identification and elimination of PI animals, (ii) enhancement of immunity through vaccination, and (iii) improvement of biosecurity to prevent exposure of susceptible cattle [244]. PI cattle are considered the most important source for BVDV and are central in the epidemiology of the virus. Ideally, detection of PI cattle should occur before entry into a herd, and all new herd additions including breeding bulls should be screened. In herds using a controlled breeding season, all calves, replacement heifers, open cows, and breeding bulls should be tested simultaneously before the beginning of the breeding season [244]. If pregnant dams are present, they should be segregated and their calves tested as soon as possible. All newborn calves should be tested as soon as possible to prevent exposure of susceptible animals. Because PI calves can be born to acutely infected or PI cows, the dams of PI calves must be retested.

When attempting BVDV control on a herd level, diagnostic assays should have a high sensitivity to detect BVDV, use an easily obtainable sample, and be highly specific for PI status. For this purpose, antigen-capture ELISA or IHC assays utilizing skin biopsy samples (ear notches) are well suited. While various diagnostic tests are available to detect infection with BVDV, not all available tests are applicable to each clinical problem. Additionally, genetic and antigenic variation of BVDV might affect the performance of detection assays [245]. Therefore test selection should correspond to the status of each herd, the tests available in different diagnostic laboratories, and economic considerations. Lack of rational use of detection methods hampers efforts in solving production problems caused by BVDV [246]. Principally, all available diagnostic assays can be grouped into methods that culture BVDV in tissue cells, detect antigen in diagnostic specimens, amplify the RNA genome of BVDV, or detect the presence of antibodies in serum or milk [246].

Virus Isolation

The isolation of BVDV from various specimens, including serum, buffy coat, nasal swabs, and tissue samples, remains the "gold standard" of diagnosis [246–248]. For BVDV diagnostics on live animals, white blood cells are the preferred sample for virus isolation as there is no interference by serum neutralizing antibodies and the virus is highly cell-associated. Tissues of the lymphoid system including spleen, thymus, and lymph nodes are the preferred sample from postmortem examinations or aborted fetuses [246]. BVDV grows readily in various cell lines, including those of other species, and this may result in difficulties when using BVDV-contaminated cell cultures [249–251]. Successful culture of cytopathic BVDV strains is indicated by vacuolation and cell death within a few days of virus inoculation. For non-cytopathic strains, immunofluorescence or immunoperoxidase staining techniques are utilized to visualize cellular infection with BVDV [252, 253]. Virus isolation may also be used as a herd screening test in the form of the immunoperoxidase monolayer assay [254]. Although economical, this test uses serum as the diagnostic sample and is not ideal for detection of acute infections or testing of cattle less than three months of age that have acquired colostral immunity. Failure to detect PI cattle with the immunoperoxidase monolayer assay has been reported, although at an extremely low rate of detection error [255].

Antigen Detection

Direct detection of BVDV antigen allows more rapid and economic testing of samples compared with virus isolation. Antigen detection can be performed by immunofluorescence, IHC, and ELISA techniques. In general, the analytical sensitivity and specificity of antigen detection assays are less than those of virus isolation, which accommodates the detection of PI cattle rather than of acute infections. In contrast to the traditional laboratory identification of PI cattle using two positive virus isolations performed three weeks apart, commercially available ELISA kits and IHC are used to identify PI cattle with a single

sample [256]. Several studies have demonstrated excellent results for antigen-capture ELISA and IHC for use as screening tests for PI cattle [257–260]. False-positive results after antigen-capture ELISA and IHC testing due to acute BVDV infections are rare but have been previously reported [261]; however, the antigen-capture ELISA and IHC tests can be utilized to test calves before waning of colostral immunity and after vaccination with modified-live BVDV vaccines which usually interfere with other assays [262–264]. Antigen detection is most frequently performed on ear-skin biopsies, but various samples including tissues, whole blood, serum, milk, and nasal swabs are suitable after appropriate sample preparation [246]. Unlike other BVDV tests, antigen-capture ELISAs cannot produce accurate results when pooled in more than two samples [265]. In addition to laboratory-based ELISA tests, a chute-side individual sample ELISA test is available, which can be used on ear-notch or serum samples to detect PI animals in 20 minutes [266].

RT-PCR

The description of the genomic sequence of BVDV and the development of suitable probes and commercially available kits has enabled the widespread use of RT-PCR as a routine diagnostic method [267]. The technique is highly sensitive and can be utilized for various diagnostic samples including skin (ear notches), milk, urine, tissues, serum, whole blood, semen, and embryos [268–273]. Although RT-PCR techniques can be used on diagnostic specimens that are fixed in formalin, RNA fragmentation occurs and reduces assay sensitivity [274].

The sensitivity of RT-PCR exceeds that of virus isolation, and the assay can detect the viral RNA of a single viremic sample in a pool of 100 negative sera [275]. The ability to pool samples such as ear notches, milk, or serum for RT-PCR BVDV testing offsets the initial greater cost of the assay and allows economical screening for PI animals. Pooling strategies using skin biopsies or blood samples rather than bulk-tank milk do not abolish the need to collect a sample from each animal in the herd [276]. In milk, BVDV was detected by RT-PCR in samples that were serially diluted to 1 : 640 [277]. Using ear notches, one study reported detection of one positive sample in a pool of 100 negative samples; however, the optimal number of samples used for pooling has been controversial, and pools of 30 samples to 100 samples have been recommended [271, 276, 278]. The economic and competitive benefits of sample pooling decrease when BVDV prevalence rates exceed 3% [279]. Testing initial pools of 20 samples followed by subsequent pools of 5 samples prior to individual testing was recommended as the pooling strategy in dairy herds with greater BVDV prevalence rates [279].

Serology

The detection of serum antibodies directed against BVDV can be performed by various assays, but the ELISA and serum neutralization tests predominate and have replaced indirect immunoperoxidase or indirect immunofluorescence techniques [280]. With appropriate application, serologic tests allow the assessment of vaccine efficacy and compliance with established vaccination protocols, the detection of herd exposures to BVDV, and the association of clinical signs with BVDV infection [281]. Difficulties exist in determining the etiology of detected antibodies, as seroconversion may be the result of acute infections, vaccination, or transfer of maternal immunity through colostrum. Commercial blocking ELISA assays that detect antibodies directed against the NS3 protein of BVDV are unable to differentiate antibodies derived from natural infection or vaccination, and marker vaccines are currently unavailable for BVDV [282]. Serologic testing of previously unexposed sentinel animals can be utilized to detect the presence of PI cattle in herds [283]. However, the analysis and comparison of serologic titers do not allow a clear distinction between PI-free herds and those with PI cattle [284].

Testing Strategy

Identification and elimination of PI cattle is the single most important step in the control of BVDV infection; however, the expense of control strategies should not exceed the cost associated with infection and disease. Whole herd testing using proper diagnostic tests is an effective method to identify and eliminate PI animals from cattle operations but may not be economically beneficial if the prevalence of BVDV in such operations is low [285]. Consequently, the strategy of testing should be developed based on the estimated prevalence of BVDV in each herd. When the prevalence of BVDV in a given herd is determined to be low (<0.47), strategies that include testing of new additions, newborn calves, aborted fetuses, and sick/dead cattle are probably more cost-effective than whole herd testing. In contrast, when herd prevalence is estimated to be greater (>0.47), whole herd BVDV testing is economically beneficial [285]. A two-test strategy could maximize the value of individual animal testing for identification of PI cattle especially when testing animals of high genetic value [261, 285]. Antigen-capture ELISA and IHC ear-notch tests can be confirmed

with RT-PCR or virus isolation (VI) in blood samples 30 days after initial testing [261].

Vaccination

In the USA, BVDV modified-live and inactivated vaccines are widely available, most commonly in the form of multivalent vaccines containing other respiratory and reproductive pathogens. While modified-live vaccines stimulate a broader immune response in vaccinated animals, are less expensive, and provide some protection after administration of a single dose, inactivated vaccines are utilized when safety concerns exist. However, failure to administer the necessary second booster dose during initial immunization can result in breakdown of BVDV control programs using inactivated vaccines [286].

Because of the antigenic diversity of BVDV strains and failure of some animals to adequately respond to immunization, vaccination efficacy cannot be 100%. Therefore vaccination programs must be part of a control strategy that includes removal of PI animals and measures of biosecurity [287]. Traditionally, only BVDV 1 strains were used in vaccine production, but the antigenic diversity between both BVDV species has prompted the inclusion of BVDV 2 in most vaccines. Studies have documented protection from BVDV 2-induced disease in cattle vaccinated with BVDV 1-containing modified-live or attenuated vaccines [288–290], but antigenic diversity of BVDV and resulting failure of cross-protection continues to be of concern. A meta-analysis concluded that multivalent vaccines containing BVDV 1 and BVDV 2 provide better protection against heterologous BVDV strains when compared with monovalent vaccines [291].

While protection of each individual animal from BVDV-induced disease is the desirable goal of vaccination programs, the increase in herd immunity and prevention of viral spread following accidental introduction are more reasonable objectives in BVDV control. Effective vaccination against BVDV should prevent viremia and spread of the virus to target organs. Additionally, vaccination should protect against clinical disease, fetal infection, abortion, and generation of PI calves. Identical immune mechanisms are stimulated to achieve these goals; however, the greater humoral and cell-mediated immune protection induced by modified-live BVDV vaccination has proven to be more effective in reducing morbidity and mortality compared with vaccination with inactivated vaccines [292]. Additionally, results from several studies demonstrated that modified-live vaccines are superior to inactivated vaccines in protecting susceptible cattle against abortion, fetal infection, and generation of PI offspring following experimental infection with BVDV [291, 293–295].

Cattle should be vaccinated to afford greatest protection during an anticipated exposure to BVDV. Therefore adequate humoral and cell-mediated immunity should be present during weaning, commingling, arrival to feedlots, and prebreeding [296]. The presence of high BVDV-specific antibody titers before experimental challenge/exposure to BVDV is associated with reduction of viremia and clinical signs [297–299]. In contrast, calves arriving at the feedyard with low antibody titers against BVDV and other respiratory pathogens have decreased economic returns, increased morbidity rates, and increased treatment costs [296, 300]. Increased morbidity rates and treatment costs were associated with inappropriate timing of vaccination close to delivery of calves or lack of a booster dose in programs using inactivated vaccines, emphasizing the value of adequate preconditioning programs [300]. Commonly, preconditioning programs as implemented in cow-calf herds require weaning of calves 45 days prior to shipment, and include dehorning, castration, bunk-training, and the administration of anthelmintics and vaccines. Calves vaccinated at 30 and 17 days before shipment to a feedlot using a multivalent modified-live vaccine did not become viremic or shed BVDV when exposed to PI penmates [301]. While modified-live vaccines are able to rapidly confer clinical protection and protected calves from experimental challenge as early as three to five days after vaccination under field conditions, complete protection cannot be expected this promptly [302, 303].

When planning a BVDV vaccination protocol for calves, blocking of protective immune responses by the presence of maternal antibodies is of concern, especially in early-weaned calves [304]. Two-week-old calves vaccinated with a modified-life vaccine in the presence of high titers of colostral antibodies were not protected from heterologous BVDV-induced disease at four months of age, in contrast to calves BVDV-seronegative at vaccination [305]. Additionally, considerable variation between individual maternal antibody titers exists, causing difficulty in estimating the ideal time of initial vaccination in calves [306, 307]. However, different investigators have recently demonstrated that vaccination of young calves in the face of maternal antibodies can result in cell-mediated and humoral anamnestic immune responses and protection from BVDV-induced viremia and disease [297, 298, 308, 309]. The results from these studies suggest that vaccination in the face of maternal immunity can be efficacious when early exposure to BVDV is expected [297, 298, 310].

In cow-calf herds, vaccination against BVDV should prevent fetal infection and birth of PI offspring, thus interrupting the endemic cycle of BVDV. Protective immunity should be achieved at least 30 days before

breeding and vaccinations should not be administered in the immediate prebreeding season to avoid adverse effects of vaccine strains on fertility [311]. In various studies, experimental intranasal challenge of vaccinated dams resulted in protection from fetal infection of up to 100%; however, complete protection was often not demonstrated [312–316]. A lower extent of protection was observed in other studies when vaccinated pregnant cattle were exposed to PI cattle, which is considered a more rigorous and realistic challenge model [293, 317–319]. While prolonged and complete protection from development of PI calves was demonstrated in a study using PI cattle as the challenge model [317], under field conditions success of vaccination to prevent fetal infection depends on various factors including timing of vaccination, nutritional and immune status of animals, cross-protection of vaccine and field strains, and number of vaccinations administered before breeding.

Biosecurity

Adequate biosecurity measures based on a practical and effective plan to identify and eliminate BVDV infected cattle are a key management factor in the prevention of introduction and transmission of BVDV in cattle herds [320]. Biosecurity measures should include isolation and testing of all purchased cattle and their offspring, using semen only from negative bulls, quarantining, testing of animals leaving the herd for shows, and separation of young stock from adults [142, 320]. Testing of all embryo transfer recipient cattle and embryo transfer collection supplies such as fetal bovine serum (FBS) is also important.

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63

Infectious Agents: Schmallenberg Virus

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Introduction

In August 2011, an unknown disease observed in dairy cows in North Rhine-Westphalia (Germany) close to the Dutch/German border was first described [1–3]. At the same time, a similar case was reported from the Netherlands. Diseased cows displayed non-specific symptoms such as fever, diarrhea, and decreased milk production [4]. In late 2011/early 2012, lambs were born that showed different degrees of malformation [5]. Laboratory tests were all negative for classical bovine endemic and emerging viruses such as bovine viral diarrhea virus 1 & 2 (BVDV 1 & 2), bovine herpesvirus 1 (BHV1), foot and mouth disease virus (FMDV), bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV), Rift Valley fever virus (RVFV), and bovine ephemeral fever virus (BEFV). Blood samples were collected from animals with clinical signs and subjected to metagenomic analysis. The results revealed a novel orthobunyavirus [1]. Because the virus was found in a pooled sample from cows from a dairy farm near the German town Schmallenberg, North Rhine-Westphalia, the new virus was named "Schmallenberg virus (SBV)" [1]. Since its first detection, the virus quickly spread all over Europe, and then continued to spread into Asia and Africa. North-, Latin-, and South America are still considered SBV free. Where SBV came from and how the virus was first introduced is still not known [3]. However, since its emergence, SBV has had significant economic impact on the cattle and small ruminant industries in countries where SBV is endemic due to direct (e.g. reduced prolificacy, malformed or dead offspring) and indirect (trade restrictions on animals and semen) losses [3].

Etiology

SBV is part of the Simbu serogroup of the genus *Orthobunyavirus* within the family Peribunyaviridae (order Bunyaviridae). Akabane virus (AKAV) [6, 7], Aino virus [8, 9], and Shamonda virus [10, 11] cause similar symptoms as SBV and are widespread in Oceania, Australia, Africa, and Asia. SBV is an enveloped virus with three negative-sense single-stranded RNA segments (small, medium, large; Figure 63.1) [12] with presumably limited antigen drift, high genetic stability, and low mutation rate [13, 14]. Only the M segment coding for two surface glycoproteins seems to be variable [3].

SBV is relatively instable in the environment or the vectors, as it does not survive outside its natural hosts for a very long time [15]. Also, it is susceptible to standard disinfectants such as 70% ethanol as well as glutaraldehyde and formaldehyde [15]. Moreover, infectivity is significantly reduced or lost at 50–60 °C for at least 30 minutes [15].

Hosts

Ruminant Species

SBV primarily infects ruminants, which includes all domestic species, i.e. cattle, sheep, and goats, but has also been found in wild and exotic ruminants such as alpaca, llama, elk, water buffalo, bison, various species of deer, mouflon, chamois, and camelids [3]. Wild ruminants may play a role in SBV transmission. However, clinical disease has not been confirmed in either wild or exotic animals [3].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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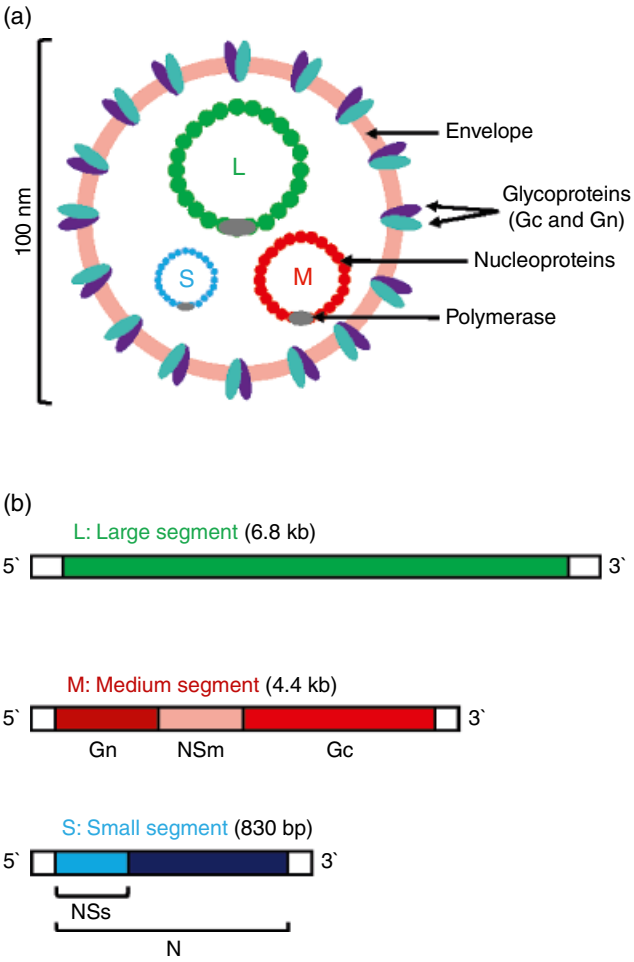


Figure 63.1 (a) Schematic model of Schmallenberg virus virion morphology with the three (-)ss circular RNA segments. (b) Maps of the three genomic RNA segments representing the distinct ORFs (L, M, S) encoding for five viral proteins. *Source*: Images courtesy of Hossam El-Sheikh Ali and Yatta Boakari.

Non-Ruminant Species

There is no evidence that SBV can infect humans. In one study conducted by the Robert Koch Institute in Berlin, Germany, and in another conducted by the National Institute for Public Health and the Environment in Bilthoven, the Netherlands, personnel working in infected sheep and goat flocks as well as in cattle barns, and the responsible veterinarians, were tested for antibodies against the virus [16, 17]. None of the tested people was positive for virus-specific antibodies. However, there are several other species that have tested SBV positive either serologically or for virus RNA. These include wild boar, dog, onager, zebra, elephant, and babirusa [3]. Clinical disease does not occur, except in one report of an SBV positive puppy that had developed torticollis and degenerative encephalopathy [18]. Domestic pigs experimentally infected with SBV transiently seroconverted, but SBV-RNA was not detected and no clinical disease was observed [19]. There is one report of SBV infection in horses from Iran, but caution is required when interpreting this result as, for example, cross-reactivity with other Simbu group viruses endemic to this area may have occurred [3, 20].

Distribution and Seroprevalence

The geographical origin of SBV is still unknown [3]. The SBV epidemic in Europe occurred quickly within a very short time period, i.e. 2 1/2 years [3]. Almost all European countries are now affected [1, 5, 21–31]. Spread continued into Russia [32] as well as neighboring countries such as Azerbaijan [33], China [34], and Turkey [35], and into Arabian (Iran [20]; Lebanon [36]) and African countries (Mozambique [37]; Namibia [38]; Ethiopia [39]). No cases of SBV infection have yet been reported from North-, Latin-, or South America.

After initial herd exposure, SBV has been demonstrated to reemerge in several European countries, albeit at lower levels compared to the first outbreak, likely due, at least in part, to the virus's ability for overwintering [40–48]. Pregnant ewes infected with SBV between days 45 and 60 of pregnancy have shown to harbor SBV in the placenta for at least 100 days post infection, which might explain overwintering in cold seasons [3, 49]. Overwintering may also occur in midges through transovarial transmission and in adult midges that are less sensitive to low temperatures such as those of the *Culicoides obsoletus* group [3, 50].

Three years post first detection, i.e. until March 2014, SBV was observed on 1,478 cattle farms as well as in 973 sheep and 53 goat flocks in Germany [51]. Generally, the prevalence of SBV infection in dairy herds is high, and higher than in sheep and goats [3]. Initial studies conducted in the Netherlands and Belgium in 2012 revealed a prevalence at farm level of 95.5 and 99.76%, respectively [52, 53]. Moreover, the intra-herd prevalence was 86.3% in the Dutch study [52]. At the same time, Spain reported a prevalence of 70.6% [54] and Turkey of 39.8% based on slaughterhouse material collected between 2006 and 2013 [55]. In a recent study from Ethiopia [39] from 2018, a prevalence of 56.6% was reported. A high between-herd seroprevalence of 98.03% was also found in sheep flocks in the Netherlands [56]. Within a flock, 58.7–84.31% of the sheep were found seropositive [56, 57]. In goats, the intra-herd prevalence was, for example, 40.68% in Belgium, 36.7% in the northwest part of Germany, and 43.8% in Lower Saxony, Germany [56–58]. In Turkey, slaughterhouse material was found to be SBV positive in 1.6% of sheep and 2.8% of goats [55].

Epidemiology

SBV is transmitted by biting midges (*Culicoides* spp.). The virus was detected in several *Culicoides* spp. (e.g. *C. obsoletus*, *Culicoides dewulfi*, *Culicoides chiopterus*, *Culicoides scoticus*, *Culicoides punctatus*, *Culicoides nebeculosis*, *Culicoides imicola*) across Europe [27, 59–62]. As with other aboviruses, a SBV transmission cycle starts with midges biting and sucking blood from an SBV-infected ruminant host. SBV then replicates within infected midges to be at transmittable levels (extrinsic incubation period, which takes 9–41 days) [63], and if infected midges then bite non-infected naïve animals, they transmit the virus via saliva. An intrinsic period follows (this takes 2–6 days) [1]. Mosquitos and black flies do not seem to be able to transmit SBV [64, 65].

Vertical transmission of SBV, i.e. through the placenta into fetuses of an infected viremic animal, is another route of infection. This route of infection causes congenital malformations to occur (see section "Pathogenesis and Clinical Signs"). The exact time window of pregnancy when animals are susceptible for vertical transmission is assumed to be between days 28 and 60 of gestation in small ruminants [66] and days 80 and 150 in cattle [7], similar to what is seen with AKAV. Moreover, assuming that SBV behaves like AKAV, infections outside these susceptible time windows may still cause effects such as embryonic/fetal death, but congenital malformations are then unlikely to occur [67].

Horizontal transmission through animal contact is unlikely, although experimentally subcutaneously inoculated cows were found virus-positive in fecal, oral, and nasal specimens [68]. In contrast, orally SBV inoculated cows did not become infected [68], and sentinel cattle kept in the same compartment as inoculated cows remained virus-negative and seronegative [68].

SBV can be detected in semen of infected bulls [69–71]. For instance, bulls subcutaneously inoculated with SBV exhibited viremia two to four days post inoculation, and highest amount of virus was detected between days four to seven post inoculation [71]. In contrast, experimentally infected goat bucks did not shed SBV in semen [72]. The role of venereal transmission of SBV therefore remains to be investigated further.

Pathogenesis and Clinical Signs

SBV pathogenesis is still not well defined. If a naïve pregnant animal is bitten by an infectious midge within the window of susceptibility and subsequently develops viremia, SBV may colonize and replicate in placentomes. However, efficient replication seems to be dependent on the maturity of the placentomes. For instance, extraembryonic material and lamb organs were more often SBV positive in sheep if experimentally infected at day 60 compared to day 45 of gestation [73]. As shown for AKAV, the susceptibility of embryos/fetuses to become infected may also be dependent on gestational stage, as no pathology was detected within the first three weeks of gestation in small ruminants and the first two months in cattle [3, 7, 66].

Generally, there are only weak or unspecific clinical symptoms in the adult pregnant and non-pregnant domestic ruminant animal associated with SBV, such as fever (up to 41 °C), decreased milk production, and diarrhea [3, 4]. Symptoms may last for up to six days (concurrent with the time period of viremia that also lasts for up to six days) [3, 4]. SBV can affect reproduction in cattle as abortions, stillbirths, and mummified fetuses have all been observed, as has reduced fertility (increased embryonic mortality, more returns and inseminations/cow, short gestations) [3, 74–77]. Similar SBV-attributed effects have been documented in sheep [74, 78–80] and goat [58, 74] flocks. However, clinical signs may also be lacking, as observed in experimentally infected goats and bucks [72]. The stage of fetal development may be of importance; ovine embryos have shown to be more susceptible before the blood–brain barrier is developed (i.e. before days 50–60 of gestation) [3, 81].

Clinical disease is most evident in calves, lambs, and goat kids after in-utero infection at the critical stage of pregnancy, causing congenital malformations belonging to the "arthrogryposis–hydranencephalia–syndrome (AHS)" (Figures 63.2 and 63.3) [3]. AHS is presented with one or more of the following malformations: arthrogryposis, vertebral malformations, brachygnathia inferior, ankylosis, torticollis, and scoliosis. In addition, malformations of the central nervous system (CNS) can occur, including hydranencephaly, porencephaly, hydrocephalus, cerebellar hypoplasia, and micromyelia [82–84]. Most of the malformed newborns are stillbirths. Survival is, however, possible, but affected offspring are then usually non-viable. Since there is indication that SBV has tropism to the musculoskeletal as well as CNS of developing embryos (with neurons in the cerebral cortex, brainstem, and spinal cord being targeted cells) [3, 81, 85], SBV infected calves and lambs may be born with unspecific symptoms that include inability to suckle and weakness in lambs [5, 86], as well as hypertonicity, hyperreflexia, depression, blindness, strabismus, and inability to stand in calves [23]. Those newborns are unviable and an immediate reason for euthanasia. Moreover, as reported for sheep, malformations may cause dystocia that would require obstetrical intervention, and also obstetrical injuries of, for example, the uterine wall [87].

(a)



(b)



Figure 63.2 Calf (a) and lamb (b) congenitally infected with Schmallenberg virus born with severe arthrogryposis of all four limbs, torticollis, kyphosis, and skeletal muscle hypoplasia. The lamb also presented without brachygnathia inferior. *Source:* Images courtesy of Dr. Benjamin Schade, Bavarian Animal Health Service, Poing-Grub, Germany.

SBV has also been shown to reduce white and red blood counts in experimentally infected calves, but this is certainly not specific enough to be utilized as a means for diagnostic testing [88].

Histological findings of the CNS included cerebral lesions and cerebral dysplasia associated with dilated lateral ventricles and a reduction of gray and white matter [89]. Viral nucleocapsid protein was detected in CNS neurons together with a moderate, non-purulent encephalitis, lymphohistiocytic vasculitis, and perivasculitis [89]. Cerebellar dysplasia and hypoplasia with either disorganization of the cerebellar cortex or multifocal to diffuse reduced thickness of the molecular layer and a reduction of Purkinje neurons may occur [89]. Skeletal muscle hypoplasia was associated with a decreased number and diameter of myofibrils occasionally in conjunction with cross-striation and fatty replacement [83, 90].

Diagnosis

SBV can be detected by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) or by virus isolation using different cell lines [3]. RT-qPCR is certainly the most versatile method, as it would allow different specimens to be analyzed. Specimens of malformed neonates such as brain stem, placenta, spleen, meconium, or amniotic fluid are appropriate [3, 91]. Native as well as processed (extended, frozen) semen can be analyzed [69–71, 92, 93]. In blood, the best time for virus detection would the period of viremia (i.e. within the first week of infection) using an ethylenediaminetetraacetic acid (EDTA) stabilizer or serum.

(a)



(b)

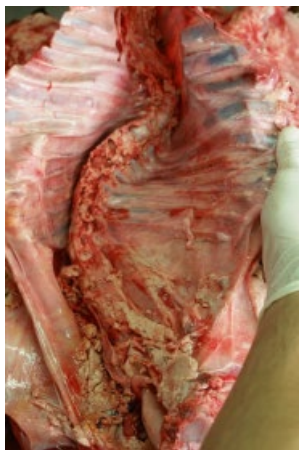


Figure 63.3 Calves congenitally infected with Schmallenberg virus born with hydrocephalus (a) and scoliosis (b). *Source:* Images courtesy of Dr. Benjamin Schade, Bavarian Animal Health Service, Poing-Grub, Germany.

SBV-specific antibodies can be detected by virus neutralization, indirect immunofluorescence test, or enzyme-linked immunosorbent assay (ELISA) (there are several commercial antibody ELISA kits available). Among all tests, virus neutralization seems to be the most sensitive test and more highly specific than the S-segment-based ELISA tests [94–96]. Antibodies can be detected in blood serum of adults [97] and milk of lactating females [98], and in different liquid specimens of ideally malformed stillborn or born-alive offspring (e.g. in blood, precolostral serum) [99–101]. Interestingly, antibody titers in milk were higher than in serum of individually collected cows [102], and were also highly predictable in bulk tank milk samples, making the latter an ideal matrix for surveillance programs [103].

Control and Prophylaxis

There is currently no treatment available for SBV-caused disease [3]. Control of SBV infection can only be achieved by vaccination, but the availability of vaccines varies by country. In Europe, there are three vaccines available to be used in both sheep and cattle: Bovilis® SBV (Intervet UK Ltd, Milton Keynes, UK), SBVvax® (Merial SAS, Lyon, France), and Zulvac SBV® (Zoetis, Parsippany, NJ, USA). All three vaccines contain inactivated virus. According to the labels, sheep are to be vaccinated at four months or older only once, while in cattle two vaccinations are required at an age ≥ two months and again three weeks later. A clear antibody response can be usually observed three weeks after the first (sheep) or second (cattle) vaccination. While it is not yet known how long protection lasts, it is assumed to be ≥12 months. Currently, there is no commercial vaccine available for use in goats.

Another preventative measure is to use repellents and pyrethroids directed against the vector, i.e. biting midges, thus reducing the risk of transmission to susceptible animals, but its sustained effect has been questioned based on a recent case control study [3, 104].

Another approach is to breed animals in colder times of the year (e.g. in late autumn or winter) when vector activity is low, in order to reduce transmission at times when fetuses are most susceptible to infection. However, this approach may have serious negative consequences to breeding management practices which may then override possible benefits by moving the breeding period, especially in areas with seasonal production systems [3, 78].

To what extent it is possible to reduce some of the risk factors, e.g. related to confinement (i.e. outdoor-kept animals are more susceptible then those confined indoor) [3], remains to be elucidated.

Finally, management practices regarding replacement animals may be employed to facilitate vector-induced infection before first breeding. Considering that immunity after natural SBV infection lasts for two to three years, those "naturally immunized" animals will then be protected at the time of first pregnancy. However, as recently summarized, this method by itself is not likely to be reliable, and a combination of different measures is required to reduce the risk of SBV infection in domestic livestock [3].

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64

Infectious Agents: Bovine Epizootic Abortion

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Introduction

Epizootic bovine abortion (EBA), commonly referred to as "foothill abortion," was first recognized in the early 1950s in California [1]. EBA is characterized by late-term abortions, or birth of weak calves, delivered by dams grazed in foothill regions of California, Nevada, and southern Oregon during their first and second trimesters of pregnancy. The disease has been, and continues to be, the leading cause of abortion in beef cattle in the state of California; estimates are not available for Oregon and Nevada [1–4].

Etiology

Multiple quests to identify the etiologic agent of EBA span 50 years. These efforts produced a number of candidate microbes as being the causative agent including a chlamydia, an uncharacterized virus (C-type-like particles), *Borrelia coriaceae*, and an uncharacterized spirochete [5]. Development of a reproducible method for experimental disease transmission in 2000 facilitated efforts to identify the etiologic agent. Inoculation of naïve pregnant heifers at ~100 days' gestation with cryopreserved fetal thymus homogenates, derived from select EBA-diseased fetuses, provided a reliable mechanism to predictably transmit the pathogen to the developing fetus with concomitant development of classical EBA. Antibiotic susceptibility studies were employed using disease-transmission experiments in pregnant heifers; aggressive treatment at the time of heifer infection with penicillin and/or tetracycline abrogated subsequent development of fetal disease, providing strong evidence that the etiologic agent was a prokaryote [5]. Application of molecular biology techniques to diseased fetal necropsy tissues ultimately identified the agent in 2005 as being a deltaproteobacterium. 16S rRNA sequences derived from the pathogen established an 89.4% homology to that of *Polyangium cellulosum* (i.e. *Sporangium cellulosum*), a member of the order Myxococcales [6]. The only other mammalian pathogen currently recognized in this class of prokaryotes is *Lawsonia intracellularis*, a deltaproteobacterium primarily associated with enteritis in pigs. The bacterial agent of EBA (aoEBA) has recently undergone additional characterization and been coined *Pajaroellobacter abortibovis* [7]. A revised 16S rRNA phylogenic analysis between *P. abortibovis* and *S. cellulosum*, taken together with the fact that *P. abortibovis* infects arachnids and is a mammalian pathogen, warrants it being in a genus separate from *Sorangium* [7].

Diagnosis

EBA was historically diagnosed with a combination of unique fetal pathology, elevated levels of immunoglobulin in fetal serum, and history of the dam being grazed in areas considered endemic for the disease during pregnancy [2, 3]. Gross and microscopic pathology associated with EBA is typically dramatic. Aborted fetuses are usually in excellent condition (not autolyzed) and present with a variety of gross abnormalities that can include extensive ascites with excessive fibrin (Figure 64.1a) resulting in a distended abdomen, swollen liver, enlarged spleen and lymph nodes (Figure 64.1b), skin lesions (Figure 64.1c), and petechial hemorrhages on mucosal membranes (oral cavity, tongue, and eyelids) (Figure 64.1d) and in the thymus (Figure 64.2a). Microscopically, lesions are widespread and have a vascular orientation characterized by infiltration with lymphocytes and mononuclear phagocytes [2, 3]. Focal necrotizing lesions may be present in organized lymphatic tissues and

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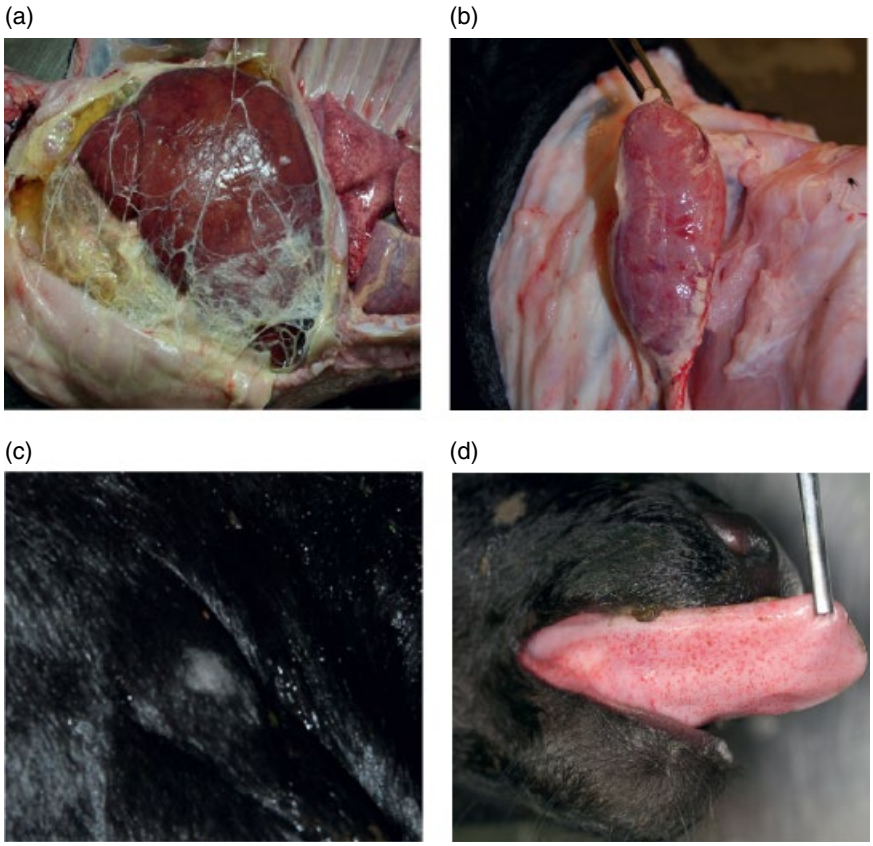


Figure 64.1 Gross changes in *P. abortibovis*-infected fetuses: (a) fibrinous peritoneal effusion and enlarged nodular liver; (b) enlarged prescapular lymph node; (c) skin lesion; (d) tongue with extensive petechiation of ventral lingual mucosa.

pathognomonic lesions occur in the thymus, characterized by depletion of cortical thymocytes and infiltration with macrophages [3] (Figure 64.2b).

Microbiological diagnosis of EBA continues to be hampered by an inability to culture the causative agent. While limited short-term replication of *P. abortibovis* has been observed in primary cultures of macrophages derived from fetal lymphoid necropsy tissues, substantive propagation of the bacterium in either primary or established cell lines has not been possible; all attempts to culture *P. abortibovis* on synthetic media have also failed [5]. Polymerase chain reaction (PCR) assays, both traditional and TaqMan-based, have been developed and target the 16S bacterial ribosomal gene [6, 7]. Both assays can be used to identify the presence of *P. abortibovis* in necropsy tissues of infected fetuses presenting with classical EBA pathology. The TaqMan is the assay of choice when identifying low levels of bacteria in necropsy tissues derived from fetuses suspected of being infected, such as fetuses presenting with equivocal or non-classical EBA pathology and weak calves that may or may not suckle. The TaqMan assay is also preferable for identifying *P. abortibovis* in the vector as only the occasional infected tick will have sufficient levels of bacteria detectable by a standard PCR [8, 9].

Routine bacterial histologic stains do not demonstrate *P. abortibovis* in fetal tissues, so a modified Steiner silver staining technique applied to the distantly related deltaproteobacterium *Lawsonia intracellularis* was used to microscopically visualize *P. abortibovis* in sections of formalin-fixed necropsy tissues. Steiner stain and immunohistochemistry applied to thin sections of formalin-fixed necropsy tissues (Figure 64.3a,b) and immunofluorescence applied to impression smears and/or homogenates of fresh tissue are the current methods of choice for microscopic diagnosis of EBA [10, 11].

A fluorescent antibody-based serologic assay has recently been validated for diagnosis of EBA [12]. Assay specificity is 100% with 95% sensitivity when cutoff criteria for a positive test were assigned at a titer of ≥1000. The assay has proven extremely useful in providing a timely diagnosis, especially in cases in which the majority of *P. abortibovis* has been eliminated by the fetal immune system. This serologic assay has also been useful in assisting veterinarians and their beef-producer clients in rural regions that do not have ready access to diagnostic laboratories as a means of diagnosing the disease by simply submitting a sample of body fluid such as blood or ascites in lieu of the entire fetus.

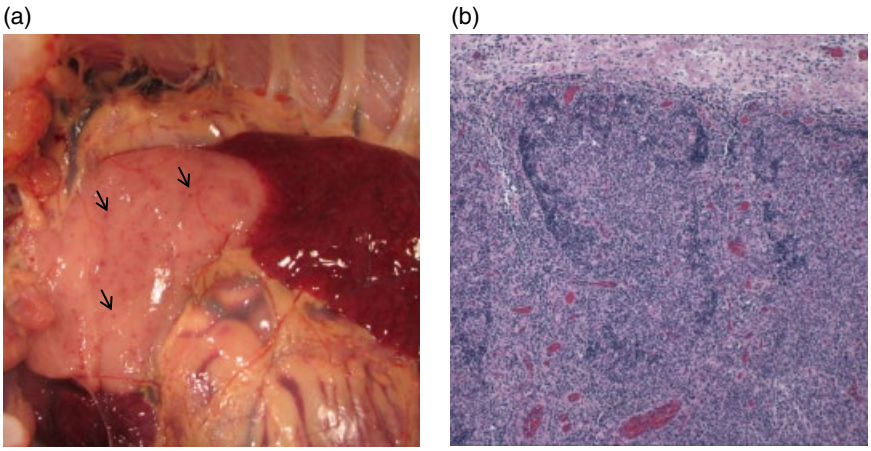


Figure 64.2 Gross and histologic changes of thymus in *P. abortibovis*-infected fetuses. (a) Thoracic thymus with petechiation. Note the petechial hemorrhages indicated by arrows. (b) Thymus exhibiting lobular atrophy with depletion of cortical thymocytes and extensive lymphohistiocytic infiltration of lobule medullary regions and the interlobular septae. Hematoxylin and eosin stain.

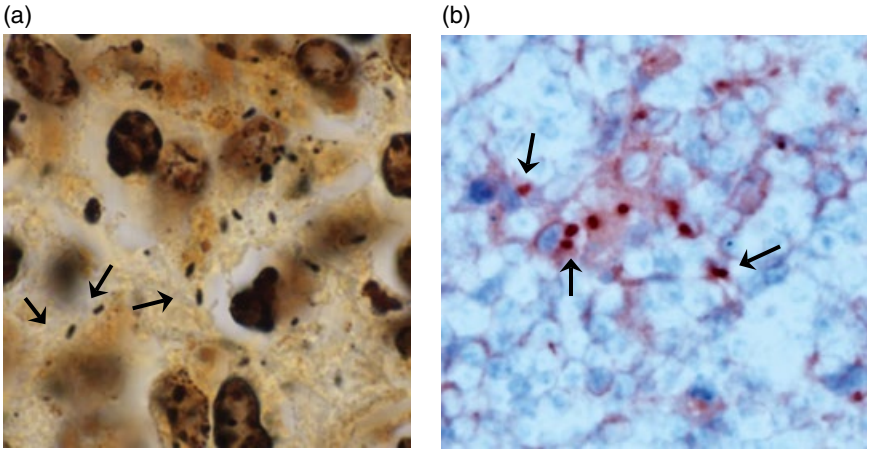


Figure 64.3 (a) *P. abortibovis*-infected thymus, modified Steiner silver stain. Note the numerous intracytoplasmic 2-μm bacterial rods indicated by arrows. (b) *P. abortibovis*-infected thymus, immunohistochemistry. Note the numerous bacterial rods indicated by arrows.

Pathogenesis

P. abortibovis apparently gains entrance to the developing fetus across the placenta from the infected dam. The dam, whether infected by tick bite or experimental inoculation, presents with no overt clinical signs. Reactions to experimental tick bites recorded in historical communications were most likely a response to contaminating microbes and/or tick saliva. The fetus has a "window" in development in which it is susceptible to an infection that will ultimately lead to fetal disease. This window is poorly defined but susceptibility is generally believed to be from conception through second trimester of gestation. Susceptibility early in gestation (<60 days) is not known. While the pathogen does not appear to impact conception, the possibility that it may induce early embryonic death in a small percentage of infected animals is under investigation. Infection of the fetus is a slow process, with definable lesions not becoming evident for two to three months [3]. The bacterium appears to replicate intracellularly in histiocytes as determined by immunohistochemistry (Figure 64.2); replication is extremely slow (approximately one day) when observed in short-term cultures of macrophages derived from fetal necropsy tissues. The intracellular nature of the infection, vascular orientation of lesions throughout the fetus, and slow and persistent development of disease would all be consistent with the hypothesis that lesions are immunologically mediated [3]. Slow growth of the bacteria is undoubtedly responsible for slow dissemination throughout the body and lesion development coinciding with development of immunologic maturation of the fetal immune system.

Pregnant sheep do not appear to be susceptible to foothill abortion; unpublished studies in which pregnant

ewes were infected in the first trimester resulted in the birth of healthy lambs. These latter studies would be consistent with the hypothesis that the bacteria replicate and disseminate slowly, and in the case of sheep the gestational period is too short to develop immunologically mediated lesions and abortion. Mule deer (*Odocoileus hemionus*) serve as an important host to the tick vector, but there is no evidence of EBA occurring in this species. Their gestation period is intermediate between sheep and cattle (six to seven months) and may be too short to allow for disease development. Alternatively, regular exposure to the organism through tick bites may confer immunity, and in those instances when abortions occur, fetuses would be rapidly scavenged, leaving no evidence. Multiple attempts to produce disease in immunocompetent laboratory animals, such as mice and rabbits, have failed.

Mice with severe combined immunodeficiency disease (SCID) are susceptible to infection with *P. abortibovis*. C3H-*scid* mice develop evidence of wasting at seven to eight weeks following infection and require euthanasia two to three weeks later [11]. The only obvious gross lesion is an enlarged spleen that harbors large numbers of infectious bacteria. Cryopreserved *P. abortibovis*-infected SCID mouse spleen cells are an excellent source of bacteria to predictably transmit infection to pregnant heifers. Unlike bacteria derived from the bovine fetus, recovered organisms are not opsonized and appear to have excellent viability. The absence of EBA-like lesions in immunodeficient infected mice would support the hypothesis that the fetal bovine lesions are immunologically mediated as suggested above [11].

Transmission

Observations that the distribution of the *Ornithodoros coriaceus* tick could be superimposed upon the distribution of foothill abortion precipitated studies to establish them as potential biological vectors [4, 8, 9]. This unusual soft-shelled tick (commonly called the Pajaroello tick) carries a colorful past as Native Americans in California feared its bite over that of a rattlesnake. They believed a second or third exposure to this tick bite would result in death. The tremendous pain and swelling of appendages that sometimes occurs following sequential Pajaroello tick bites is assumed to be an extreme allergic reaction to the tick saliva and/or associated contaminating microbes. An unusual and classical experiment was conducted in which naïve pregnant heifers were divided into two groups in an area considered endemic for the disease. One half of the heifers were maintained in tree houses with the tree bases tightly wrapped with metal to interfere with the potential ascent of crawling insects, and the other half were maintained on the ground below. All tree-housed heifers produced healthy calves, while foothill abortion occurred in the land-based animals. Studies were subsequently conducted in which wild-trapped ticks were experimentally fed on pregnant naïve heifers, further demonstrating disease transmission [4, 5]. The *O. coriaceus* tick has been collected in Mexico, California, Nevada, and Oregon (Figure 64.4), but foothill abortion has only been described in the above-mentioned three US states [8, 9, 13, 14]. Cattle ranching practices in Mexico may well be responsible for a lack of diagnosis of foothill abortion in this country; aborted fetuses can be rapidly scavenged in free-ranging cattle herds, making diagnosis unlikely.

Published studies have been directed at better understanding the vector–pathogen relationship using field-trapped ticks and PCR/TaqMan diagnostics to identify the presence of *P. abortibovis*. *O. coriaceus* can be readily collected in the field using dry-ice traps in the duff beneath brush and trees that have previously served as bedding areas for wildlife and/or livestock. These studies confirmed the extensive distribution of *O. coriaceus* in foothill regions of southern Oregon, central and western Nevada, and California [8, 9, 14]. Application of diagnostics to DNA extracted from ticks demonstrated a highly variable rate of infection with *P. abortibovis*. A positive result obtained by standard PCR was rare [9]. Data obtained using the more sensitive TaqMan suggest that infection rates are typically less than 20% but may exceed 50% (examples of high infection rates may be a mathematical artifact due to low tick numbers). The authors suggest that those ticks that are positive by TaqMan but not standard PCR may not be capable of biological transmission of the bacteria to cattle [9]. This would be supported by studies where experimental feeding of hundreds of ticks per heifer results in definitive disease transmission approximately 50% of the time [4, 5]. Efforts to better characterize the tick as a vector of *P. abortibovis* demonstrated that the bacterium was preferentially located to the salivary gland of field-collected ticks [9]. The source of this bacterium is currently unknown; possibilities being considered include soil or the presence of a mammalian/avian reservoir. A blood meal is required prior to each molt (progressive instar stages result in increased tick size) and maturation into either a male or female. The authors hypothesize that if ticks are infected while feeding, the multiple blood meals required during the process of maturation would increase the incidence of infection in larger (more mature) ticks. Studies suggest little difference in infection rate in nymphs, males, and females and therefore appear to contradict the concept of infection though a blood meal [9].



Figure 64.4 Published reports of trapped *O. coriaceus* (blue circles). Recent select collections of ticks were subjected to TaqMan-based detection of *P. abortibovis*; blue circles with red centers represent positive results. The majority of tick collection sites were sampled prior to the identification of *P. abortibovis* and associated PCR-based diagnostics. Tick collections in Mexico are based on historical literature and their locations roughly approximated. *Source:* © DeLorme. Topo North America™ 10.

Control

Control of foothill abortion has largely been limited to management practices. One approach is minimizing exposure of naïve pregnant cattle to tick-infested habitat during the window of gestation in which the fetus is susceptible to infection. Another approach is to intentionally expose naïve cattle to the tick prior to breeding such that immunity can be established; historically, EBA was not as prevalent prior to the current practice of breeding young heifers (less than two years of age). Such practices can sometimes modestly reduce losses; however, the reduction in foothill abortion is often offset by inefficient use of rangelands and increased costs associated with altered breeding and grazing strategies. An experimental vaccine has been extensively field-tested in excess of 60,000 heifers and the product became commercially available in September, 2020. Experimental and field testing of the vaccine have demonstrated the vaccine to be safe with apparently >95% efficacy. The vaccine is a cryopreserved live virulent bacteria derived from spleen cells of *P. abortibovis*-infected SCID mice; the vaccine cannot be safely administered to pregnant cattle. Extensive, though unpublished, data have demonstrated that the live bacteria have no adverse health effects, though animals clear the bacteria very slowly (30–40 days post vaccination). Naïve heifers vaccinated (infected) less than 30 days prior to conception can conceive but then can transfer *P. abortibovis* to the developing fetus, with a resulting fetal resorption/abortion three to four months later. Thus, vaccinated heifers should not be bred until 60 days post-vaccination. Limited experimental data have demonstrated solid immunity for a minimum of three years; this is consistent with field trial date that would suggest that administration of the vaccine to heifers in areas endemic with EBA will result in life-time immunity.

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65

Infectious Agents: Neospora

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Introduction

Neosporosis is caused by the protozoan parasite *Neospora caninum*, an obligate intracellular parasite found in dogs and cattle as well as other species. True prevalence of infection is difficult to determine since published reports tend to be specific for a particular region. However, it is estimated that *Neospora* infects 10–20% of cattle and is responsible for 20% of abortions worldwide [1]. This parasite was first described in dogs having encephalomyelitis in Norway [2] in 1984 and was previously misidentified as *Toxoplasma gondii* until 1988, when the new species, *N. caninum*, was described [3]. The first report of bovine abortion associated with *Neospora* infection was from a New Mexico dairy in the USA in 1989 [4]. Both domesticated [5, 6] and wild [7–10] canids can serve as definitive hosts, while cattle [1], deer [11], and chickens [12] are intermediate hosts. However, there is some doubt that the red fox is truly a definitive host [13]. Although transmission can occur both horizontally [14] and vertically, vertical transmission is the primary source of infection in cattle [1]. The economic impact of *N. caninum* is the result of numerous factors including abortions, culling, decreased milk production, increased calving interval, and increased veterinary costs [15]. Global losses are estimated at USD $1.3 billion and in excess of $600 million in the USA annually [16].

Epidemiology

N. caninum has been identified worldwide and is considered a major cause of abortion in cattle [17]. Globally, 15–20% of dairy cattle and 10–20% of beef cattle have been found seropositive for *N. caninum* [1]. Surveys in California [17] the Netherlands [18], and New Zealand [19] indicate that approximately 20% of all aborted bovine fetuses submitted to diagnostic laboratories tested positive for this infection. The estimates of *Neospora* infection in US dairy cattle, based on serology, are variable but appear to be in the range 10–20% and ranges from 5 to 98% of cows in individual dairy herds. Many animal species test seropositive for *N. caninum* but do not exhibit clinical signs [1]. Due to the lack of one gold standard diagnostic test [20] numerous testing methods are utilized for identifying infection such as recognition of tissue cysts on histological examination, finding oocysts in feces [6], or identification of the parasite by immunohistochemical staining [21] or polymerase chain reaction (PCR) [22].

Life Cycle

The life cycle of *N. caninum* is depicted in Figure 65.1. *N. caninum* has a facultative heteroxenous life cycle, meaning that the organism may use more than one host during its life cycle [23, 24]. Unsporulated oocytes (10–14 μm) are shed in the feces of definitive hosts (i.e. dog or wild canid) [25]. The oocytes sporulate within 24 hours to the infective form. Each sporulated oocyte contains two sporocysts, and each sporocyst contains four sporozoites. When consumed by cattle or a wide range of other warm-blooded animals which can serve as intermediate hosts, eight sporozoites are released into the gastrointestinal tract for each oocyte consumed [1]. The sporozoites differentiate into tachyzoites (5–7 μm), which subsequently invade the epithelial cells of the gastrointestinal tract. The tachyzoites replicate rapidly via endodyogeny [26]. Endodyogeny is a form of asexual reproduction that involves an unusual process where two daughter cells are produced inside a mother cell, which is then consumed by the offspring prior to their separation [27]. Replication occurs rapidly within cells and tachyzoites may spread hematogenously within mononuclear phagocytes [28] to infect many cell types including neural cells, vascular endothelial cells,

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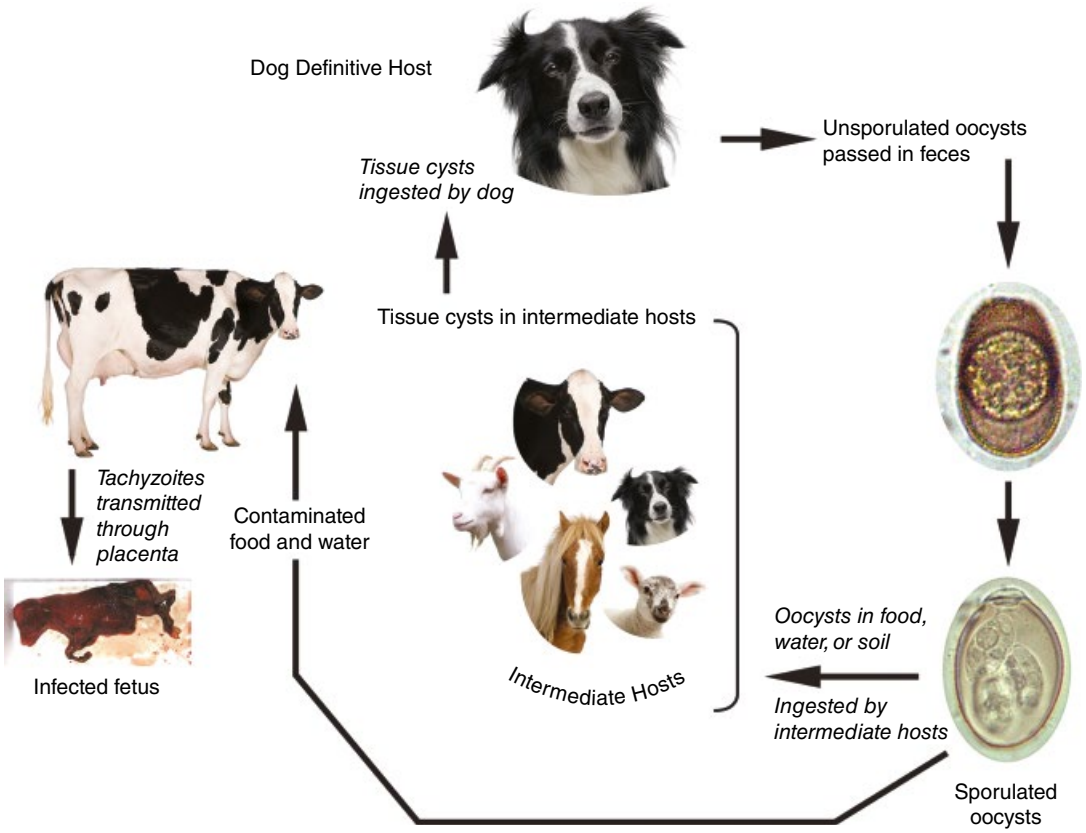


Figure 65.1 Life cycle of *Neospora caninum*. *Source:* Reproduced with permission from [8].

myocytes, hepatocytes, renal cells, alveolar macrophages, and placental trophoblasts [29–31]. Tachyzoites differentiate into bradyzoites which are the slowly replicating encysted stage of the parasite. Each cyst may contain hundreds of bradyzoites [24]. In congenitally infected bovine fetuses and calves, tissue cysts are found in the brain and spinal cord [32]. A few thin-walled cysts have been found in skeletal muscle of two naturally infected two-day-old calves [33]. Bradyzoites are thought to persist for the duration of the host's life and be responsible for persistent infection, although tissue cysts have not been observed in histological sections of naturally infected cattle older than two months [24, 29]. Bradyzoites are believed to reactivate and differentiate back into tachyzoites, thereby establishing persistent infection [24]. Bradyzoites can be differentiated in tissue sections from tachyzoites by labeling with a specific antibody [34]. The cycle continues when tissue cysts shed by the intermediate host (cattle) are consumed by the definitive host (canids) [1].

Transmission

Neospora can be transmitted vertically or horizontally, with both routes being vital to long-term survival of the parasite [24]. Vertical transmission takes place when tachyzoites migrate transplacentally from dam to fetus during pregnancy [1, 25]. Among all known microbes in cattle, *N. caninum* is one of the most efficiently transmitted parasites transplacentally [1]. The terms "endogenous transplacental transmission" and "exogenous transplacental transmission" have been used to more precisely describe the origin and route of fetal infection (Figure 65.2) [23]. Endogenous transplacental transmission occurs in a persistently infected dam when the infection crosses the placenta and infects the fetus [23]. The endogenous form has a higher rate of associated abortion [35]. Exogenous transplacental transmission occurs when a previously non-infected dam ingests infective oocysts while pregnant and her fetus subsequently also becomes infected in utero.

Vertical transmission can result in abortion but in most cases the calf is congenitally infected but remains asymptomatic with no evidence of deleterious effects on subsequent calf health [36]. If the infection occurs during the second or third trimester when immunocompetence of the fetus is greater, the most likely outcome will be a live-born but persistently infected calf [24]. Once infected, cattle are presumed to be infected for life [37] and females can transmit the infection to successive generations [38, 39]. Although not every pregnancy results in transmission of the disease, it has been reported that the rate of transmission is as high

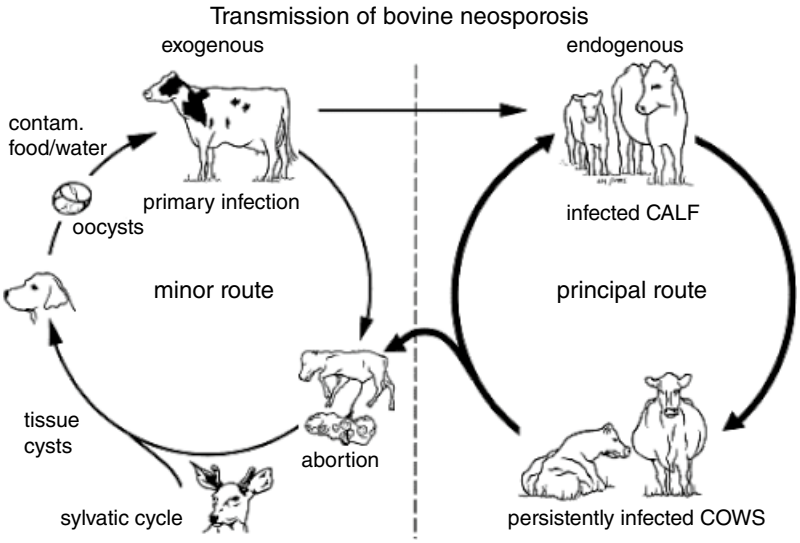


Figure 65.2 Transmission of bovine neosporosis. Oocysts are produced by the canine definitive host and their subsequent ingestion by a susceptible pregnant cow leads to infection of the fetus (exogenous transplacental transmission). Liveborn infected heifer calves would be expected to remain infected into adulthood when they, in turn, may pass infection to their fetus (endogenous transplacental transmission). Spread of *N. caninum* in this second way is the principal route whereby the parasite is propagated in a herd. *Source:* [26, 48, 49].

as 75–100% [16]. In the case of exogenous transplacental transmission, the number of oocysts ingested by the dam and stage of gestation influence pregnancy outcome [25, 40]. There is a report indicating that in persistently infected cattle, vertical transmission is more efficient in younger than older cows [41]. Also, transplacental infection may be more likely to occur in dams that were themselves prenatally infected compared with postnatally infected dams [42]. The risk of abortion was positively correlated with *N. caninum*-specific antibodies in individual animals [43] and severity of fetal *Neospora*-associated lesions also increased with higher maternal seropositivity [44]. Cows with high antibody titers (≥400) showed higher vertical transmission frequency (94.8%) than cows with low antibody titers of 25–200 (14.8%) [45]. Occasionally, a seronegative dam will give birth to a seropositive calf [46, 47]. This can occur when the dam has a long-standing infection with low antibody titer and she was wrongly diagnosed as uninfected [1].

Horizontal transmission is less common than vertical transmission [16]. The ingestion of sporulated oocysts from the environment is the only demonstrated natural mode of postnatal infection in cattle (Figure 65.2) [48, 49]. Both domestic and wild dogs are frequently implicated in horizontal transmission. This is attributed to canids consuming infected bovine placentas or fetuses and subsequently defecating in the environment where the oocytes are ingested by cattle [50]. There has been no horizontal cow-to-cow transmission reported at this time [38]. Experimentally, calves may become infected through ingestion of milk contaminated with tachyzoites [51, 52] and *N. caninum* DNA has been detected in colostrum of seropositive cows [53]. However, it remains doubtful that lactogenic transmission occurs naturally [50]. Since *N. caninum* DNA was reported in fresh and frozen semen from naturally infected bulls [54], the possibility of venereal transmission was investigated. Intrauterine inoculation of nine heifers with 107 *N. caninum* tachyzoites reacted with seroconversion and a specific interferon (IFN)-γ response. Also, *N. caninum* DNA was demonstrated in the blood and tissues of all nine heifers [55]. However, the numbers of tachyzoites used to test the venereal route of infection are higher than those previously found in semen of naturally infected bulls, which range from 1 to 10 organisms per milliliter associated with the cell fraction of semen [56, 57]. Therefore it seems unlikely that venereal transmission is significant under natural conditions.

Pathogenesis of Abortion

Neosporosis is primarily a disorder of the fetoplacental unit that occurs subsequent to maternal parasitemia resulting from either exogenous or endogenous infection [24]. In the case of exogenous infection, the ingested oocytes excyst in the small intestine and presumably release sporozoites which parasitize the maternal intestinal epithelium and transform into tachyzoites which, in turn, multiply in the mesenteric lymph nodes [24]. The tachyzoites are released into the blood and have been detected in the leukocyte fraction [28] of naturally infected cattle. The parasitemia results

in dissemination throughout the body including the gravid uterus. The endogenous (vertical) route of transmission is most common [38, 58]. It is suspected that latent infections are reactivated due to the immunosuppression associated with mid-gestation pregnancy [59]. It is also reported that endogenous transplacental transmission is more likely in cattle that were themselves infected in utero [60, 61].

The mechanism by which *N. caninum* actually causes abortion is a topic of ongoing investigation and multiple hypotheses have been proposed. First, since the parasite is able to establish itself in the caruncular septum before crossing to the fetal placental villi [62, 63] the resultant damage may result in fetal death due to placental insufficiency [24]. Placental insufficiency, because it causes fetal stress and release of adrenocorticotropin, could also cause premature delivery of either viable or compromised calves. Second, infection by *N. caninum* stimulates cell-mediated immune responses associated with cytokine release from the damaged placenta, resulting in fetal rejection by the maternal immune system culminating in fetal expulsion [24, 64, 65]. Finally, the placental damage may result in endometrial prostaglandin (PG)$F_{2\alpha}$ release causing luteolysis and abortion as has been demonstrated in goats with toxoplasmosis [66]. However, cows with high *Neospora* antibody titers have higher plasma progesterone levels throughout gestation than seronegative cows or cows with low antibody titers [67]; in a study in which exogenous progesterone was administered during the mid-gestation period, the risk of abortion increased dramatically in cows with high levels of antibodies against *N. caninum* [68]. It seems most likely that all three of the proposed mechanisms play a part in inducing *Neospora*-associated abortion in cattle.

The first-trimester fetus seems particularly susceptible to damage by invading *N. caninum* since it is not immunocompetent [69] and unlikely to survive infection. In the middle trimester the fetus may be able to mount an immune response to *N. caninum* challenge, although the response may not be robust enough to preclude fetal death [59, 70, 71]. By the third trimester the fetus is capable of mounting an immune response of sufficient magnitude that it is likely to survive *N. caninum* infection, leading to birth of clinically normal but infected calves. In beef cows experimentally infected with *N. caninum* at various times during gestation by either subcutaneous or intravenous routes, transplacental infection rate increased with gestational age and cows infected during later gestation generally delivered normal but infected calves [40].

In cows experimentally infected with *N. caninum* the most severe lesions are found in the placenta and the fetal brain [40, 70]. Lesions consist of a non-suppurative inflammatory response [63]. The associated inflammatory cells have been shown capable of producing INF-γ [59], which suggests fetal death may be more a consequence of the maternal immune response rather than direct effects of the parasite [59]. At the time of placental invasion the parasite also enters the fetal bloodstream, with a predilection for the central nervous system [63], where it is located perivascularly [30], and in younger fetuses causes widespread destruction of neuropil with a minimal inflammatory response [72]. When fetuses later in gestation become infected with *N. caninum* the tissue reaction is more moderate, with areas of necrosis restricted to small foci surrounded by intense inflammatory infiltrate in brain tissue [18, 73] and sometimes mild meningitis [74]. Fetal infection with *N. caninum* also causes characteristic lesions of inflammation and necrosis in tissues such as liver and heart [18].

Clinical Signs

N. caninum infection causes abortion in both beef and dairy cattle. Abortion may occur from three months of gestation to term but typically occurs at five to six months of gestation [4, 18, 75, 76]. Possible outcomes of infection include: fetal death followed by resorption [77]; mummification [78]; autolysis [79]; stillbirth [80]; live birth with clinical disease [79]; and clinically normal birth but persistently infected [36]. Fetuses dying in utero between three and eight months are usually expelled with evidence of moderate autolysis, while those dying before five months of gestation are more likely to mummify [24]. Experimental infection of pregnant cows at various points during gestation showed that parasitemia during the first 10 weeks resulted in fetopathy and resorption of the fetal tissue 3 weeks after infection. Infection at 30-weeks of gestation resulted in birth of asymptomatic congenitally infected calves [60]. The period in between appears as most likely for *N. caninum*-induced abortion.

In *Neospora*-infected calves clinical signs have been reported only in animals less than two months of age [81]. Infected calves may be born underweight, unable to rise, and with neurologic signs [81]. Hindlimbs and/or forelimbs may be flexed or hyperextended and the calf may be ataxic, have diminished patellar reflexes, and experience loss of conscious proprioception [81]. Exophthalmia has been observed in some cases [81].

Within herds, *Neospora*-associated abortions may be sporadic, enzootic, or endemic [4, 17, 30, 82]. In comparison with endemic abortion, epidemic abortion storms are less common [83]. It appears that most sporadic and endemic abortions in cattle due to neosporosis are the result of reactivation of chronic infections because *Neospora*-seropositive

cows are at twofold to threefold higher risk of abortion than are seronegative cows [76, 78]. Reactivation of a chronic *Neospora* infection may occur as a result of factors that cause immunosuppression. We have observed a *Neospora*-associated abortion storm in a dairy herd concomitant with an outbreak of coliform mastitis [46]. In herds with endemic *Neospora*-associated abortions there is often a positive correlation between serostatus of the dam and her daughters, indicating that the major route of transmission is vertical [84, 85].

Epidemic abortion outbreaks (abortion storms) have been defined as those in which over 10–15% of at-risk cows abort within a four to eight week period [78, 84, 86]. There is epidemiological evidence that epidemic *Neospora*-associated abortions may be caused by the postnatal infection of naive cattle via exposure to oocyst-contaminated feed or water, with up to 57% of pregnant dairy cows aborting over a few weeks to months [84, 87, 88]. However, in an epidemiological investigation of abortion storms in the Netherlands, the authors concluded that the abortion storms appeared to be induced by factors causing recrudescence of *N. caninum* infection in chronically infected animals rather than being the result of a recent introduction [86].

Diagnosis

Definitive diagnosis of neosporosis is challenging and relies on numerous diagnostic techniques. There are serologic assays available to test the maternal serum and fetal fluids (serum, fluid from body cavities) for *Neospora*-specific antibodies [89]. Serology of fetal fluids is useful after five months of gestation as the fetus becomes immunocompetent but sensitivities reported varied from 50 to 84% [90]. Immature fetal immunocompetence and a short interval between infection and death can all lead to a false-negative result [90]. The presence of antibodies does not definitively confirm *Neospora* as the cause of abortion since clinically normal calves may also have congenital antibodies [76].

Serology of the dam can aid in diagnosis of *Neospora*-associated abortion. Sera obtained within two weeks of abortion generally have the highest titers and then titers decline and may be undetectable 60 days after abortion [89, 91]. Seropositive cows do not display detectable clinical signs of infection and most deliver healthy congenitally infected calves. Therefore, seropositivity has a high predictive value for congenital infection in cows but not for abortion [92] (although there is a two to threefold increased probability of abortion of compared with seronegative cows [76]). High antibody levels persist for prolonged periods in some cows [91] or may increase again in subsequent gestations [90]. Seronegativity in a mother cow or heifer does not exclude *N. caninum*-associated abortions [93].

PCR has been utilized to detect parasite DNA [94], but it is not always found [29]. In a Swiss study, *N. caninum* was detected by PCR in the brain tissue of 21% of all aborted fetuses. Microscopic lesions indicative of cerebral protozoal infection were detected in 84% of PCR-positive fetal brains [93].

Histopathology of fetal tissues can be examined to identify characteristic lesions and parasites [95]. Multifocal inflammation and necrosis of the heart, brain, liver, and skeletal muscle are common and tachyzoites are found primarily in the brain [18, 90] Lesions can be found in the placenta but are not particularly valuable as diagnostic aids [90]. Even in grossly autolyzed or mummified fetuses it is possible to find suspect lesions, especially in the brain [90]. On necropsy, gross lesions are not frequently seen.

To positively identify parasites associated with lesions, immunohistochemistry is commonly employed [17, 32]. In one study, in which *N. caninum* tachyzoites were identified by immunohistochemistry performed on tissues taken from calves with confirmed neosporosis, 85% of the brains were positive, 14% of the hearts, and 26% of the livers [18]. Significant differences between epizootic and sporadic abortion cases with regard to positivity on immunohistochemical examination were found only in the liver, where tachyzoites were more frequently found and in higher numbers in epizootic cases than from sporadic cases [18]. One must bear in mind that positive results of any diagnostic tests without the presence of life threatening lesions are insufficient for a definitive diagnosis [29].

In investigations of herds in which multiple abortions occurred, a tentative diagnosis can be supported by cross-sectional or herd serology in which the *N. caninum* status of aborting and non-aborting cows is compared to determine if there is an association between serologic status and abortions [91, 96]. In the case of an abortion storm, a suggested protocol would be to immediately take blood samples from all animals at risk, then take post-abortion samples to determine if seroconversion occurred [90].

Control/Prevention

A number of approaches have been employed to control neosporosis in cattle herds. These include improving farm biosecurity, test and cull programs, test and exclude from breeding, and artificially inseminating with beef semen. Biosecurity may prove beneficial to avoid introducing the parasite into a closed herd not already infected with *Neospora* [97]. Farm management practices to reduce *N. caninum* infection can include: (i) minimizing fecal contamination of cattle feed

or water by canids [1]; (ii) prompt removal of aborted bovine fetuses and fetal membranes; and (iii) limiting the introduction of infected cattle into the herd and culling infected animals. If employing the test and cull method of control, every animal must be serologically tested and those that test positive removed from the herd. Animals that test negative should be periodically retested since titers tend to wax and wane and there is a possibility of horizontal transmission, resulting in new infections. This approach can be devastating depending on how many in the herd test positive. Notwithstanding the risk of horizontal transmission, a more economic approach would be to test each animal and exclude the daughters of seropositive cows from the replacement pool. If a cow is very valuable there is always the opportunity to preserve the genetics by embryo transfer into a seronegative dam [98].

It is possible to reduce the risk of *Neospora* abortions in dairy cattle by inseminating seropositive dams with beef breed semen. This is effective because crossbreed pregnancies have a more robust placentation with higher levels of peripartum pregnancy-associated glycoprotein, which may have protective value [99]. Beef breeds in general, and the Limousin breed in particular, are more resistant to *Neospora* infection than are dairy breeds [99]. Beef cow-calf herds that manage their cows on range for summer grazing have lower seroprevalence than those that do not, while increased seroprevalence is associated with higher winter stocking density [100].

These control methods might not be economically or practically feasible on dairies or beef cattle operations [101]. Buying replacements originating from seronegative herds can mitigate the risk of introducing infected replacements into a negative herd [102]. General management efforts to reduce stress from concurrent disease, environmental and social stress, and providing an adequate wholesome ration may reduce immunosuppression and hence abortion rates.

There is accumulating evidence that some *N. caninum* infected cows can develop a degree of protective immunity against abortion and/or congenital transmission, indicating that immunoprophylaxis to prevent abortion or congenital transmission is a feasible goal [66]. A commercial vaccine is not currently available. In the future, vaccine development is likely to depend on identification of specific *Neospora* genes that may enable the production of genetically engineered vaccines.

Treatment

Currently, there are no approved practical chemotherapeutic compounds useful for treatment of bovine neosporosis [16]. Various antimicrobial agents have been tested *in vitro* [103–105] or *in vivo* in mice [106], but there is no known drug that can be used to clear *N. caninum* infection in adult cattle. However, when toltrazuril was administered to congenitally infected newborn calves the infection was eliminated, suggesting that vertical transmission in subsequent generations may be reduced by treatment of calves soon after birth [107].

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Infectious Agents: Mycotic Abortion

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Introduction

Fungi are a diverse group of eukaryotes that includes yeasts, molds, mushrooms, rusts, smuts, and the recently reclassified microsporidia. They constitute one of the five kingdoms of life (Prokaryotae, Fungi, Protista, Plantae, and Animalia) in the current classification system. Like other eukaryotic organisms, fungi have membrane-bound nuclei, membrane-bound cytoplasmic organelles, and 80S ribosomes contained within a plasma membrane. Their membranes contain sterols, namely ergosterol, which determine cell membrane fluidity and act as boundary lipid. Fungi lack chloroplasts, found in plants and some protists, and have cell walls that contain chitin, unlike other eukaryotes. Many can reproduce both sexually (teleomorphic state) and asexually (anamorphic state).

There are several valid and recognized classification schemes for the kingdom Fungi. Recent phylogenetic analysis, based on ribosomal RNA and associated genes, divides the kingdom Fungi into one subkingdom, the Dikarya including the phyla Ascomycota and Basidiomycota, and five additional phyla composed of Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Glomeromycota, and the Microsporidia [1, 2]. Taxonomy based on molecular detection of genetic sequences has had little effect on the commonly known genus and species names of fungi, but did substantially change their higher taxonomic relationships. From a classical medical perspective, pathogenic fungi can also be classified into dermatophytes, yeasts, dimorphic fungi (having two anamorphic forms), hyaline hyphomycetes (e.g. *Aspergillis* spp.), dematiaceous hyphomycetes (a disparate group of melanin-pigmented fungi), coelomycetes (anamorphic fungi that form conidia in a cavity called pycnidia), zygomycetes (aseptate fungi), and basidiomycetes (a large group of fungi including puffballs, shelf fungi, rusts, smuts, and mushrooms that bear sexually produced spores on specialized cells called basidia) [3]. Additionally, based on morphology and reproductive features, fungi have been historically classified into the Ascomycota (sac fungi), Basidiomycota (club fungi), Zygomycota (conjugation fungi, aseptate), Deuteromycota (fungi imperfecti, with no known sexual state), and the Mycophycophyta (lichens and symbiotic fungi) [4].

At a very basic level, fungi are easily separated into two major morphologic categories: the filamentous or true fungi and yeasts. Most fungi are ubiquitous saprophytes in the environment, which feed on and recycle decaying organic matter. They prefer dark moist environments in which to grow. Filamentous saprophytic fungi, represented by hyaline hyphomycetes, dematiaceous hyphomycetes and zygomycetes, are by far the most commonly encountered agents producing bovine mycotic abortion.

Mycotic Agents

A wide variety of filamentous fungi and several yeasts have been reported to cause bovine abortion [5]. Mycotic abortion is a sporadic event. The incidence of mycotic abortion varies from 2 to 20% depending on the environment, location, and time of the year and generally less than 10% of the herd is affected [6]. The most common cause of bovine mycotic abortion is *Aspergillus* spp., responsible for approximately 84% of the cases reported. Of these, *Aspergillus fumigatus* was cited as the cause in 64% of 369 cases in which only one fungal isolate was obtained in culture (Table 66.1) [7]. In

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

Table 66.1 Fungi isolated in culture from 369 US cases in which a single isolate was obtained.

Fungus	Classification/features	Prevalence (%) in 369 cases
Filamentous fungi		
Aspergillus fumigatus	Ascomycota, hyaline hyphomycetes	64
Aspergillus terreus	Ascomycota, hyaline hyphomycetes	7.3
Aspergillus flavus	Ascomycota, hyaline hyphomycetes	2.7
Aspergillus nidulans	Ascomycota, hyaline hyphomycetes	3.8
Aspergillus rugulosus	Ascomycota, hyaline hyphomycetes	<1.0
Penicillium thermophilus	Ascomycota, hyaline hyphomycetes	<1.0
Penicillium vermiculatus	Ascomycota, hyaline hyphomycetes	<1.0
Penicillium flavus var. *flavus*	Ascomycota, hyaline hyphomycetes	<1.0
Scedosporium boydii	Ascomycota, hyaline hyphomycetes	2.4
Phialophora mutabilis	Ascomycota, dematiaceous hyphomycetes	<1.0
Curvularia geniculata	Ascomycota, dematiaceous hyphomycetes	<1.0
Exophilia jeanselmei	Ascomycota, dematiaceous hyphomycetes	<1.0
Scytalidium dimidiatum	Ascomycota, dematiaceous coelomycete	<1.0
Exophiala dermatitidis	Ascomycota, dematiaceous hyphomycetes	<1.0
Absidia corymbifera	Zygomycetes, aseptate	6.5
Rhizomucor pusillus	Zygomycetes, aseptate	3.0
Rhizopus arrhizus	Zygomycetes, aseptate	3.8
Rhizopus rhizopodoformis	Zygomycetes, aseptate	<1.0
Mortierella wolffi	Zygomycetes, aseptate	<1.0
Yeasts		<1.0
Candida krusei	Ascomycota, yeast	1.0
Candida pseudotropicalis	Ascomycota, yeast	<1.0
Candida tropicalis	Ascomycota, yeast	<1.0
Candida lusitaniae	Ascomycota, yeast	<1.0
Candida glabrata	Ascomycota, yeast	<1.0

Source: Adapted from [7].

mixed fungal infections, occurring in about 11% of the cases, *A. fumigatus* was found in association with other zygomycetes in 87% of cases examined. In approximately 14% of the 369 cases examined, zygomycetes were confirmed as the sole causal agents, constituting the second most common cause of bovine mycotic abortion. Zygomycetes were responsible for 21% of the abortions when considered with mixed infections. Dematiaceous ascomycetes as a group were associated with 7.5% of these cases. Yeasts of the genus *Candida* caused 2.4% of bovine abortions [7].

Pathogenesis and Pathology

The most likely routes for exposure to fungi causing mycotic placentitis and abortion are the respiratory and gastrointestinal tracts [8]. Intrauterine inoculation of *Aspergillus* conidia has been unsuccessful in experimental induction of mycotic abortion and ascending reproductive tract infections therefore seem unlikely [9]. Moreover, mycotic abortions have been experimentally induced by intravenous administration of *Aspergillus* microconidia and the hematogenous route is thought to be the path because lesions develop initially in the placentomes [8]. Fungi may enter the circulation through the alveolar septa or through gastrointestinal lesions resulting from penetration of mucosal barriers by rumen, reticular, or omasal infections or ulcers. Once the infection is established in the placentomes, it advances laterally to the intercotyledonary spaces. The placenta becomes grossly thickened, with a leather-like appearance of the intercotyledonary spaces. There is necrosis of the cotyledons and caruncular tissue and a thickening at the margins of the caruncle, imparting a concave or dished appearance. The placenta may have dry, thick, yellow

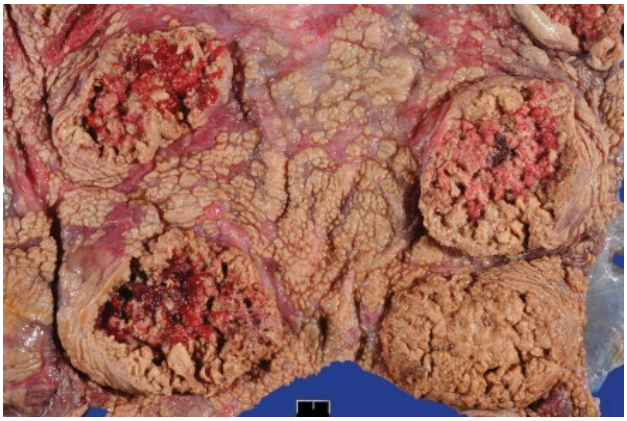


Figure 66.1 Bovine mycotic placentitis produced by *A. fumigatus*. Cotyledons are necrotic and there are thick yellow plaques covering intercotyledonary areas. *Source:* Photograph courtesy of Dr. J. Cooley.

plaques covering the intercotyledonary areas and most cotyledons when infected by *Aspergillus* spp. [10] (Figure 66.1).

A severe placental necrotizing vasculitis with thrombosis is a hallmark lesion associated with mycotic abortions. However, placental lesions with similar appearance can be produced by brucellosis and genital campylobacteriosis. Subsequently, fetal infection may occur involving the skin, lungs, brain, or liver. Fetal death is usually attributed to placental insufficiency. Skin lesions typically appear as dry, wrinkled, raised plaques with *Aspergillus* spp.; contrastingly, the fetus may appear wet and moist when infected by zygomycetes. The fetus is aborted soon after death, which occurs most commonly between six and eight months of gestation. Frequently the placenta is retained and the cotyledons remain firmly attached to the maternal caruncles, which may rupture at the peduncle. These latter events are most commonly observed with zygomycotic abortions and may lead to subsequent secondary infections [5]. Without severe uterine damage, most cows recover and have normal pregnancies later in life.

In approximately 25% of abortions caused by *Mortierella wolfii*, a zygomycete, severe post-abortion pneumonia is recognized to occur [7]. Following abortion, the caruncles and uterus can release large numbers of infective elements, resulting in an acute fungal embolic pneumonia. Cows usually die within 72 hours following the onset of clinical signs that include rapid shallow respiration with forced expiration [11]. In *M. wolfii* fetal infections, the liver and brain are commonly involved, unlike infections caused by other fungi [12].

Diagnosis

Diagnosis of mycotic abortion can be aided by gross, microscopic, histopathologic, or cultural methods of examination. Additionally, molecular detection of genetic sequences has been employed to identify specific fungal agents [13]. Definitive diagnosis is generally achieved through agreement of many of these methods. The visual demonstration of fungal hyphae and elements by microscopic methods in placental or fetal tissues and fluids combined with compatible gross lesions may confer a presumptive diagnosis. Histopathology combined with cultural or molecular methods allows for definitive confirmation of the etiologic agent responsible for abortion.

Specimens

Specimens for mycotic abortion can be used for gross examination, direct microscopic and histopathologic examination, fungal culture, and molecular detection. Specimens can include the placenta, fetus, fetal skin, fetal lung, and stomach fluid of the fetus. Because a definitive diagnosis of mycotic abortion frequently depends on agreement using multiple methods, the comprehensive specimen list presented in Table 66.1 is generally required. Specimens should be carefully selected and independently collected for each of the methods used. Extreme care should be taken to avoid contamination of specimens used for culture or molecular detection, which can easily lead to false-positive results.

The placenta is the most common and useful specimen for diagnosis of mycotic abortions. Because the lesions of mycotic placentitis may be focal and not involve some areas, the entire placenta should be submitted. The cotyledons with caruncular tissue contain the highest number of organisms and are considered to be the best specimen. The placenta will always be involved in mycotic abortion; however, the fetus or fetal tissues are not consistently affected.

Following abortion, the fetus can become easily contaminated by contact with the environment. In approximately 25% of mycotic abortions the fetal skin is infected and lesions appear as circumscribed, slightly raised, cutaneous, gray to yellow plaques. These lesions may coalesce into larger areas along the dorsum, the top of the head, and base of the tail. Although amniotic fluid is not commonly available following abortion, it will contain fungal elements when the skin is affected and transport them to other fetal areas and tissues that are not as easily contaminated [10, 14].

Stomach fluid is a commonly used specimen for culture and molecular detection when the fetus has been expelled and contaminated on its surface. Stomach fluid can also be used as an excellent specimen for direct microscopy. Likewise, the fetal lung is exposed to the amniotic fluid and commonly used to circumvent contamination of the fetal surface when diagnostic amplification techniques are employed.

Microscopic and Histopathologic Examination

Direct microscopic examination of the placenta, fetal skin, and abomasal fluid following digestion in 10–15% potassium hydroxide (KOH) can reveal fungal hyphae. Digestion of host debris is time and temperature dependent and can be quickened by the application of gentle heat. Direct visualization of fungal elements can be enhanced with the inclusion of calcofluor white in 10% KOH solution, which specifically binds cellulose and chitin of the fungal cell wall. Calcofluor white (or Uvitex B, Blankophor) fluoresces blue-white or apple-green when exposed to ultraviolet light [15–17]. Additionally, Parker permanent blue ink (10%) can be added to the KOH solution to impart contrast and aid visualization of hyaline hyphal elements. Lactophenol cotton blue dye can be used as a simple positive stain with stomach fluid specimens that do not necessarily need alkaline digestion.

The microscopic morphology and characteristics of the fungi can aid in identification and confirmation of the cultural isolate. The hyphae of zygomycetes are relatively broad (5–20 μm), thin-walled, and coenocytic (aseptate) [18]. Budding yeasts with narrow bases and broad pseudohyphae having constricted septa can indicate pathogenic *Candida* species [19]. The dematiaceous fungi causing abortions have brown to black intracellular pigment (melanin) that is easily seen. *Aspergillus* species have relatively narrow (2–6 μm) hyphae and frequent septae spanning parallel walls [10]. It is important to note that identification to the species level is not attainable through direct microscopic methods, but these methods can be quickly used to confirm a presumptive diagnosis of mycotic abortion, suggest a group of responsible fungi, and rule out other causes of abortion.

Adequate areas and portions of the placenta and fetal skin and lung should be collected into 10% buffered neutral formalin for histopathology if the whole fetus is not submitted to the laboratory. In routine hematoxylin/eosin-stained histopathology slide sections, hyaline fungal elements are poorly visualized. Therefore requests for special staining of slides using periodic acid–Schiff or Gomori's methenamine silver stain should be made if mycotic abortion is suspected.

Fungal Culture Methods and Molecular Identification

Because of the ubiquity of fungi in the natural environment, culture results should be interpreted with caution. Contamination of specimens from abortion cases is frequent. For this reason, specimens of the fetal lung and abomasal fluid or contents are commonly sought. The morphology and characteristics of the fungi recovered in culture should be in agreement with the results of direct microscopy and histopathology.

A common medium for the culture and isolation of fungi is Sabouraud's dextrose agar (SDA) with and without the addition of antibiotics. Antibiotics such as chloramphenicol and cycloheximide are commonly included in the media, such as Mycobiotic Agar (Difco), to suppress the growth of bacteria and saprophytic fungi, respectively. Care must be taken in choosing media as cycloheximide will inhibit zygomycetes. It can also partially inhibit some *Aspergillus* spp. and *Candida* spp. involved in mycotic abortions. For this reason, SDA with only chloramphenicol is recommended for the culture of fungal agents from mycotic abortions. Furthermore, zygomycetes can be difficult to culture as they do not remain viable in tissue for extended periods of time. Failure to recover these fungi when observed in direct microscopy or histopathology can be due to the toxic effects of placental or fetal autolysis [11]. Agar plates are incubated at 25–30 °C for several weeks prior to morphologic identification.

Following isolation and maturation of growth, filamentous fungi are classically identified on the basis of colonial and microscopic morphology (Figure 66.2). Speciation of the filamentous fungi can be problematic using these



Figure 66.2 Photomicrograph of a lactophenol cotton blue-stained slide of *A. fumigatus* from SDA plate culture. Note conidiophores with large terminal vesicles supporting numerous sterigmata that bear long chains of conidia. *Source:* Photomicrograph (20×) courtesy of Dr. F.W. Austin.

techniques and advanced molecular methods may be necessary. Yeasts are commonly identified on the basis of carbohydrate assimilation and biochemical testing using commercially available systems [19].

Molecular detection of specific genetic sequences obtained through polymerase chain reaction (PCR) methods is becoming commonly used in mycology reference laboratories for fungal identification. These methods can be applied to fresh tissues, tissues fixed for histopathology, or cultural isolates [13]. Identification of fungal species using unique genetic sequences can save considerable time and offer unparalleled specificity. However, because of the ubiquity of fungi in the environment and the extreme sensitivity of molecular methods, results should be used in conjunction with other methods to assure a conclusive diagnosis.

Prevention and Control

Since mycotic abortions occur sporadically and are produced by ubiquitous environmental fungi that are not contagious, the best means of control are preventative. Avoidance of moldy hay and feedstuffs and poor-quality silage can decrease the occurrence of mycotic abortion. Likewise, dark and moist environments with a high organic component favoring the growth of fungi should be eliminated. Decreasing environmental exposure to fungi by providing adequate ventilation, avoiding animal confinement, and decreasing herd density can further aid in preventing mycotic abortions. Therapy should be aimed at preventing uterine damage from retained placenta and associated secondary infections.

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Compromised Pregnancy Success Caused by Heat Stress

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Introduction: Nature of the Problem

A useful term to describe the reproductive function of a herd of cows is 21-day pregnancy rate. Conceptually, this term measures the proportion of cows eligible to become pregnant in a 21-day period (i.e. those cycling) that actually become pregnant. The 21-day pregnancy rate is the product of the percentage of cows that are inseminated and the percentage of inseminated cows that become pregnant. In an ideal situation, all cycling cows will ovulate in a 21-day period, be inseminated at estrus, and establish pregnancy after insemination. In practice, the 21-day pregnancy rate is much lower. For US Holsteins using data from 2007 to 2016, the 21-day pregnancy rate averaged across regions and seasons was 15.7% [1] whereas median 21-day pregnancy rate in herds in Québec between 2001 and 2010 was 18% [2].

Heat stress is among the most consequential changes in the environment of the cow that can reduce 21-day pregnancy rate. Estrus detection rates as low as 20% during periods of heat stress have been reported [3] and pregnancy per artificial insemination (AI) can be 10% or even lower [4]. Management practices on many modern dairies have made the impact of heat stress, while significant, not as dire as could be the case. Results of a recent analysis of seasonal variation in reproductive function among Holsteins in the USA are shown in Figure 67.1. As compared to cows inseminated in winter, 21-day pregnancy rates for cows in summer declined by 5.3 percentage points in the southeastern states of the USA, 5.6% in the southern plain states, and by lesser amounts in less-warm regions of the country. Even in the northeastern USA, however, 21-day pregnancy rate declined in the summer.

Heat stress is defined here as the sum of forces external to a homeothermic animal (mammal or bird) that acts to reduce the animal's ability to regulate core body temperature. Consequences of heat stress on the physiology and productive potential of the cow depend on both physiological changes that an animal engages to regulate body temperature and the direct negative effects of elevated body temperature on cellular function. One of the causes of reduced milk yield during heat stress, for example, is lower feed intake by cows seeking to regulate body temperature through reduced metabolic heat production [5]. A cause of reduced fertility is the embryonic death caused by effects of elevated body temperature on embryonic viability [6–8]. In this chapter, the term heat shock will be used to describe actions of elevated temperature on cellular function.

Even a small increase in body temperature can compromise embryonic survival. In lactating dairy cows, conception rate declined 6.9–12.8% for each 0.5 °C increase in uterine temperature above the mean temperature of 38.3–38.6 °C [9]. In that study, rectal temperature was about 0.2 °C lower than uterine temperature, and therefore it can be inferred that fertility declines when rectal temperature reaches no more than about 39 °C. In an earlier study, also, fertility of lactating dairy cows declined at rectal temperatures of 39 °C and higher [10].

As shown in Figure 67.2, the problem of reproductive dysfunction caused by heat stress is greater for lactating dairy cows than for dairy heifers [11, 13–15]. What makes the lactating dairy cow more susceptible to heat stress is the difficulty in thermoregulation caused by the metabolic heat production of lactation. In Wisconsin, lactating Holsteins began experiencing rectal temperatures above 39 °C when the dry bulb temperature was 25 °C, whereas heifers first developed a rectal temperature above 39 °C when the dry bulb temperature was 33 °C [12]. Another consequence of the heat production required for milk

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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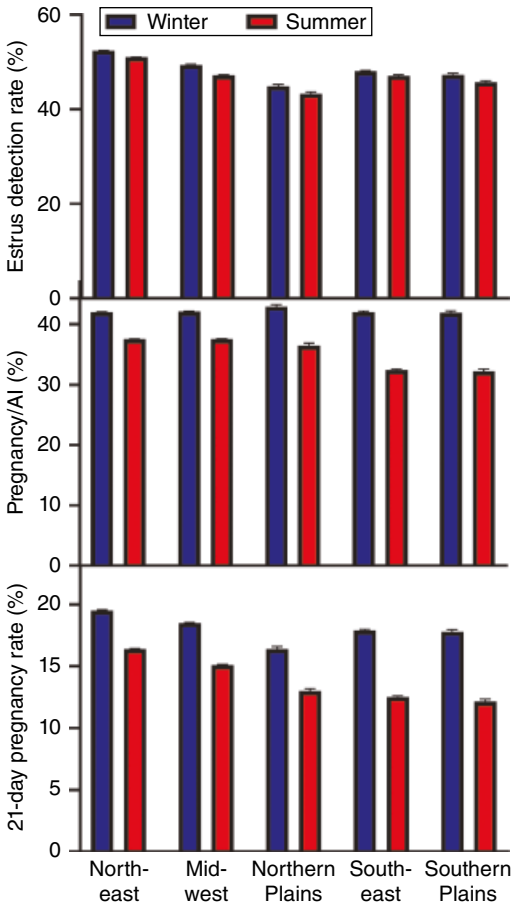


Figure 67.1 Differences in estrus detection rate, pregnancy/AI, and 21-day pregnancy rate between Holstein cows bred in summer (21 June to 21 September) and winter (21 December to 19 March) in the USA from 2007 to 2016. *Source:* Data are from [1]. Note that estrous detection rate includes cows inseminated using timed AI programs without estrus detection.

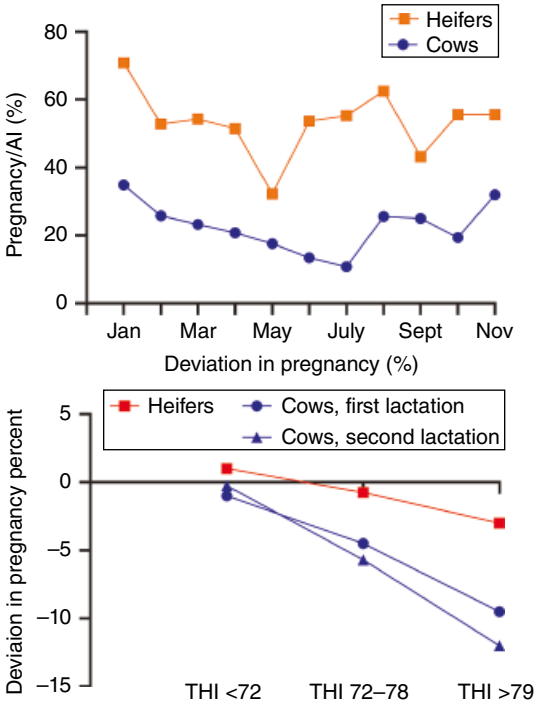


Figure 67.2 Differences between heifers and lactating cows in effects of heat stress on fertility. Data in the top panel represent the effect of month of insemination on pregnancy/AI of Holstein × Lai Sind crossbreds in Vietnam [11]. Data in the bottom panel are the deviation in pregnancy/AI percentage during July and August (pregnancy/AI minus average pregnancy/AI across the year) from Holsteins in Japan [12]. Heifers and cows were categorized based on the average temperature–humidity index (THI) during August.

synthesis is that higher-producing cows are more susceptible to infertility caused by heat stress than cows producing less milk [16–18].

Experimental heat stress of beef cattle can have large deleterious effects on pregnancy per insemination [19] and it is likely that the biology of heat stress is largely the same for all cattle. Nonetheless, heat stress is often not a large determinant of reproductive function in beef cattle. Metabolic rate is not as high as for lactating dairy cows, breeding seasons are typically not organized in the hottest time of year, and many cattle used for beef production in warm climates are genetically adapted for heat stress.

This chapter will focus on effects of heat stress on reproductive events important for establishment of pregnancy. There are also important effects of heat stress during late gestation that can affect placental function, fetal growth, and postnatal function [20] and on the fertility of the bull [21], but these aspects of heat stress are beyond the scope of the chapter. The first part of the chapter will describe what is known about the physiological and cellular mechanisms by which heat stress compromises reproductive function. The second part will address strategies for minimizing heat stress effects on reproduction.

Causes of Compromised Pregnancy Success

Estrous Behavior and Ovulation

Estrous behavior is reduced in cows exposed to heat stress (Figure 67.3). This conclusion is based on comparison of summer–winter differences in duration of estrus [22, 26], number of mounts per estrus [23], and the increase in pedometer activity during estrus [24]. Not surprisingly then, the ability of herdsmen to detect estrus is reduced during seasons associated with heat stress [1, 3, 27]. Reduced estrous behavior could be caused by reduced preovulatory concentrations of estradiol-17β in the blood [26]. It may also be that cows experience lethargy when heat

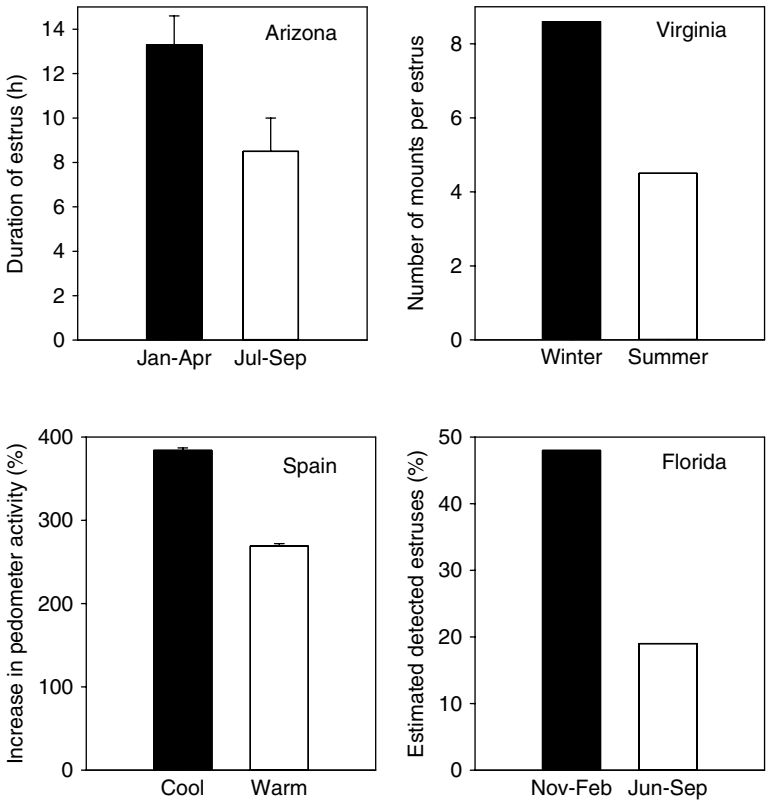


Figure 67.3 Seasonal changes in various characteristics of estrus in lactating cows including duration of estrus in Arizona [22], number of mounts per estrus in Virginia [23], increase in pedometer activity at estrus in Spain [24], and percentage of estrus periods estimated detected by herdsmen in Florida [3]. *Source:* [25].

stressed. Cows exposed to heat stress undergo several behavioral changes including increased time spent standing versus lying [28, 29] and there is evidence that prolonged periods of heat stress lead to reduced standing, possibly because of fatigue [29].

There is one report that ovulation failure can be increased during heat stress [30]. In particular, the percentage of cows that failed to ovulation after estrus was 12.4% in the warm period of the year and only 3.4% in the cool period. As will be discussed in the next section, heat stress can cause deviations in follicular development and such effects could compromise the ability of the follicle to undergo ovulation. In addition, the magnitude of the gonadotropin release caused by gonadotropin releasing hormone (GnRH) injection was reduced during periods of heat stress in a subpopulation of lactating cows (i.e. those with low circulating estradiol-17β concentrations) [31]. Thus, reduced pituitary responsiveness to GnRH could conceivably contribute to anovulation.

Compromised Function of the Oocyte

Heat stress can impinge on oocyte function during two distinct periods of oocyte development. The first is the period of follicular growth, when the oocyte experiences activation and then silencing of the genome, establishes communication with cumulus cells, and synthesizes cortical granules [9]. The second period is following ovulation when the oocyte undergoes nuclear and cytoplasmic maturation in preparation for fertilization and embryonic development.

Oocyte Damage During Follicular Growth

Damage to the oocyte during follicular growth has been demonstrated in experiments to evaluate seasonal variation in competence of oocytes to be fertilized *in vitro* and develop into blastocysts (Figure 67.4). In these experiments, which used lactating Holstein cows, the proportion of oocytes that became blastocysts after *in vitro* fertilization [32–34] or chemical activation [35] was lower in summer than winter. The seasonal effects on blastocyst formation largely represented decreased competence of cleaved embryos to develop rather than competence of the oocyte to cleave. One reason is that heat stress reduces accumulation of molecules in the oocyte important for early embryonic development and affects abundance of specific transcripts in the resultant embryo [36].

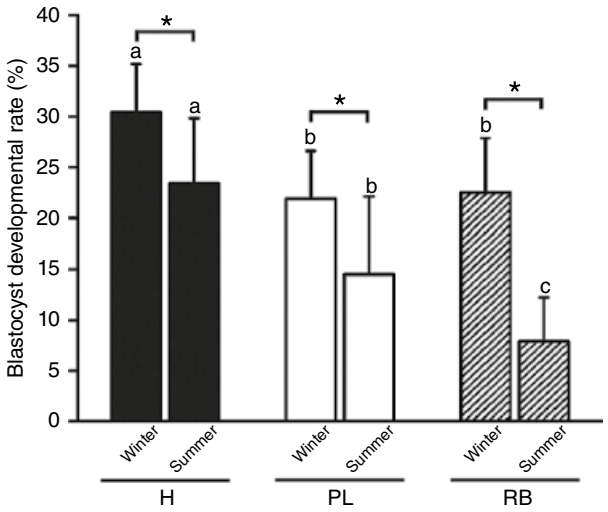


Figure 67.4 Seasonal variation in Brazil for oocyte competence for development in Holstein heifers (H), lactating cows at the peak of lactation (PL), and lactating cows classified as repeat breeders (RB). Oocytes were harvested by transvaginal aspiration and subjected to *in vitro* maturation and fertilization. Resultant embryos were cultured to the blastocyst stage. Asterisks represent differences within animal type between seasons. Letters above each bar represent differences between animal types within an individual season. *Source:* [32], © 2011, Elsevier.

Appropriate follicular development is critical to the ovulation of an oocyte competent to be fertilized and transform into an embryo capable of successful development [37, 38]. Heat stress can alter follicular dynamics by reducing growth of the dominant follicle and increasing the number of smaller-sized follicles [39–42]. Furthermore, heat stress can reduce follicular concentrations of estradiol-17β [43] and insulin-like growth factor-1 [42] while increasing follicular concentrations of androstenedione [43].

The mechanism by which heat stress alters follicular development is not well understood. Heat stress can reduce luteinizing hormone secretion [44, 45]. There may also be direct effects of elevated body temperature on steroid synthesis. Culture of cumulus cells at 40.5 °C reduced estradiol-17β production in the autumn but not in the summer or winter [43]. In the same study, incubation at 40.5 °C reduced forskolin-stimulated androstenedione production by thecal cells when cells were collected in winter but not when cells were collected in summer or autumn. Heat shock of 41 °C reduced basal and gonadotropin-stimulated synthesis of androstenedione and estradiol-17β by cultured follicular walls from the dominant follicle [46].

In some cases, heat stress can cause a reduction in circulating progesterone concentrations [47] and such a decline could conceivably contribute to a reduction in oocyte competence. This idea is based on the observation that fertility following ovulation of a first-wave dominant follicle, when circulating progesterone concentrations are low, is lower than fertility following ovulation of a second-wave dominant follicle, when progesterone concentrations are high [48, 49]. Moreover, supplementation with progesterone during the first follicular wave improved fertility [49].

Summer heat stress is followed by a gradual restoration of oocyte competence for fertility [16, 18] and oocyte competence for chemically activated development [50]. These observations are symptomatic that heat stress can affect follicular development early in the ~16-week period it takes for a primordial follicle to reach dominance [51]. Indeed, there is experimental evidence for delayed effects of heat stress on function of the follicle and oocyte. In one study [52], exposure of lactating Holsteins to heat stress affected follicular steroid production 20–26 days later. An experiment in Gir cattle (*Bos indicus*) indicated that carryover effects of heat stress can persist for up to 133 days after initiation of heat stress (i.e. 105 days after the end of heat stress) [39]. Cows were exposed to heat stress for 28 days and oocytes aspirated and subjected to *in vitro* maturation and fertilization at weekly intervals for up to 133 days after the beginning of heat stress. Heat stress did not affect follicular dynamics during the 28-day heat stress period but increased the diameter of the first and second largest follicles, as well as the number of follicles >9 mm, from days 28–49 after initiation of heat stress. Moreover, the percentage of oocytes that became a blastocyst was reduced by heat stress even at the end of the experiment at 133 days after initiation of heat stress (Figure 67.5).

Oocyte Maturation

The oocyte remains susceptible to disruption by heat stress during the process of nuclear and cytoplasmic maturation in the periovulatory period. Heat stress of superovulated heifers for a 10-hour period beginning at the onset of estrus and before insemination at 15–20 hours after the beginning of estrus decreased the proportion of embryos recovered at day 7 after estrus that were classified as normal [53]. There was no effect of heat stress on fertilization rate, so damage to the oocyte resulted in formation of an embryo with reduced competence for development.

Some of the effects of heat stress on oocyte function during maturation could be due to alterations in hormone secretion because, as mentioned previously, heat stress reduced the magnitude of the preovulatory surge in luteinizing hormone and estradiol-17β [26, 31]. In addition, exposure to elevated temperature can induce errors in oocyte maturation. Culture at elevated temperatures during maturation reduced the proportion of oocytes that completed nuclear maturation; increased the proportion with abnormal spindle formation, cortical granule distribution, depolarized

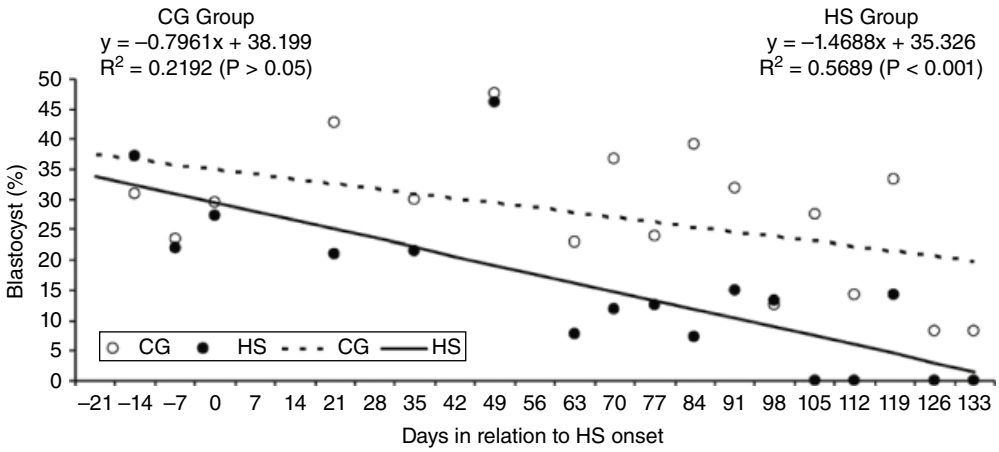


Figure 67.5 Changes in developmental competence of oocytes harvested from Gir cows that were either experimentally heat-stressed for 28 days (HS) or were maintained in a control environment (control group, CG). Oocytes were subjected to *in vitro* maturation and fertilization. Resultant embryos were cultured to the blastocyst stage. Note that competence of oocytes to become blastocysts remained lower for the HS group for up to 133 days after initiation of heat stress (i.e. 105 days after the end of heat stress). *Source:* [39] © 2008, Elsevier.

mitochondria, and apoptotic pronuclei; and decreased the proportion that cleaved and became blastocysts after insemination [54–59]. A recent experiment suggests heat shock alters histone methylation marks in the resultant four-cell and eight-cell embryo embryos [60].

Apoptosis has been implicated in disruption of oocyte function during maturation. Inhibition of apoptosis with a caspase inhibitor [55], sphingosine 1-phosphate [61], or inhibitor of ceramide synthesis [62] reduced effects of heat shock on developmental competence of the oocyte. Effects of heat shock on the oocyte also involve reactive oxygen species (ROS). Culture at elevated temperature increased oocyte production of ROS [63–65], and addition of the antioxidants cysteine [63], astaxanthin [64], melatonin [65], and retinol [66] protected the cultured oocyte from heat shock.

Effects of heat shock on the maturing oocyte, which are based on experiments conducted *in vitro* in artificial culture media, should be reevaluated in light of findings that follicular fluid contains molecules that can protect the oocyte from elevated temperature [67]. Specifically, addition of either follicular fluid or follicular fluid exosomes to oocytes undergoing maturation *in vitro* reduced the effects of culture at 41 °C on oocyte developmental competence. There are also reports that the ovary exists at a temperature about 1.1 °C less than rectal temperature [68]. If the ovary experiences local cooling, it would be unlikely to be compromised by direct effects of elevated temperature.

Fertilization Failure

Formation and maturation of spermatozoa in the male reproductive tract can be compromised by heat stress [69, 70] and there is evidence that embryos formed from sperm from heat-stressed bulls have reduced developmental competence [71]. Effects of thermal stress on the bull can be eliminated by AI. Fertilization in females inseminated artificially can still be compromised during periods of heat stress, as illustrated by results in Figure 67.6 comparing fertilization rates for lactating cows in summer and winter [12]. A major cause of reduced fertilization rate during heat stress is the result of actions of heat stress on the oocyte that were described in the section "Oocyte Maturation." The spermatozoa deposited in the reproductive tract may also be damaged by heat stress, however. Culture of spermatozoa at elevated temperatures as low as 40 °C reduced motility but not fertilizing capability [72]. At 41 °C, both motility and fertilizing ability were compromised [73, 74] through a mechanism involving mitogen-activated protein kinase 14 [74]. There may also be some long-term consequences for the embryo of being derived from a sperm cell exposed to heat shock in the female reproductive tract. There are reports in the rabbit that exposure of sperm to elevated temperature *in vitro* [75] or *in vivo* [76] resulted in abnormalities in development leading to a reduction in the number of implantation sites despite no effect on fertilization rate. More work is necessary to determine if damage to the spermatozoa by heat shock can lead to developmental abnormalities later in gestation in cattle.

Damage to the Preimplantation Embryo

When first formed, the preimplantation embryo is very susceptible to elevated temperature. A heat shock as low as 40 °C for 12 hours reduces development of zygote-stage embryos to the blastocyst stage [7]. Heat shock also reduces

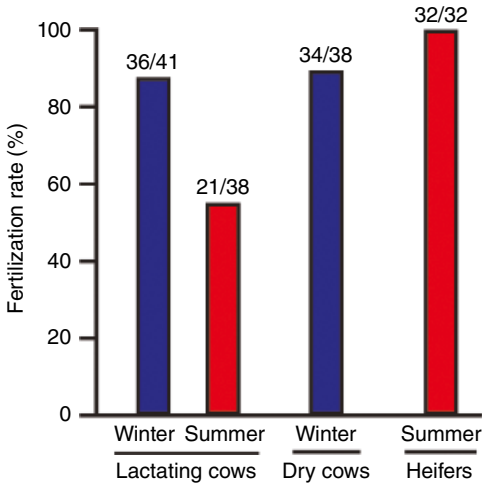


Figure 67.6 Seasonal changes in fertilization rate in Holsteins in Wisconsin. The reproductive tract of inseminated females was flushed 5 days after ovulation to recover oocytes and embryos. Fertilization rate was based on number of recovered embryos (>2-cell) divided by number of recovered oocytes and embryos (see numbers above each bar). For lactating cows there was a decrease in fertilization rate in summer compared to winter. For non-lactating animals, however, there was no difference in fertilization rate between seasons (compare dry cows in winter with heifers in summer). *Source:* Data are taken from [12].

developmental potential of embryos at the two-cell stage of development [6, 8], but thereafter the embryo becomes more resistant to heat shock. Development of four- to eight-cell embryos is compromised by heat shock but to a lesser extent than for two-cell embryos [6]. By the morula stage of development, the embryo is substantially resistant to deleterious effects of heat shock [6–8, 77]. The same phenomenon is observed *in vivo*. Exposure of superovulated cows to heat stress reduced blastocyst yield at day 8 after estrus when heat stress occurred at day 1 after estrus but not when it occurred at day 3, 5, or 7 after estrus [78].

Mechanism for Embryonic Mortality Caused by Heat Stress

The two-cell embryo exposed to 41 °C experiences alterations in ultrastructure characterized by swelling of mitochondria and movement of organelles toward the center of the blastomere [79] (Figure 67.7). Organellar movement is caused by disruption of the cytoskeleton [80]. Some of the damage caused by heat shock could be the result of increased production of ROS since, like for the oocyte, exposure of the preimplantation embryo to elevated temperature increases ROS production [8, 81]. In one experiment, effects of heat shock were greater in two-cell embryos cultured in a high oxygen atmosphere that facilitates oxidative stress [82]. In contrast, effects of heat shock were independent of oxygen tension in other studies [7, 83]. Similarly, the antioxidant dithiothreitol [82] and anthocyananin [84] have been reported to mitigate actions of heat shock, whereas other antioxidants including melatonin [81], vitamin E [85], and glutathione [86] were without embryoprotective properties.

Using Japanese Black cows, there is evidence for differences in the endometrial transcriptome between summer and winter at day 15 of the estrous cycle [87]. Accordingly, actions of heat stress on embryonic survival *in vivo* could involve effects on the reproductive tract as well as on the embryo directly. Heat stress can reduce the magnitude of the preovulatory surge in estradiol-17β during the preovulatory surge [45] and during the first follicular wave after ovulation [41] and these hormonal changes could conceivably change function of the oviduct or endometrium. So too could changes in circulating concentrations of progesterone but reported effects of heat stress are highly variable, with heat stress being associated with a decrease [88], increase [89], or no change [90] in blood progesterone. The direct effects of heat shock on oviductal or endometrial function have not been examined at temperatures characteristic of those experienced by heat-stressed cows. However, culture at 40.5 °C increased production of prostaglandin (PG)$F_{2\alpha}$ and E_2 from endometrial stromal cells [91] and PGE_2 from oviductal epithelial cells [92].

Developmental Acquisition of Thermotolerance by the Embryo

Several hypotheses have arisen to explain the process by which the embryo transitions from a state in which it is very susceptible to heat shock, at the one-cell to two-cell stage, to one, at about the 16-cell stage, where it has acquired resistance to heat shock. One possibility is that the early embryo is very susceptible to heat shock because processes required for embryonic genome activation are easily disrupted by heat shock. If this was the case, embryos exposed to heat shock at the two-cell or four-cell stage should block development at between the 8-cell and 16-cell stages; embryonic genome activation is required for development past this stage [93]. Such a preferential block at the 8- to 16-cell transition was seen in one experiment with two-cell embryos [83] but not in another where embryos were heat-shocked at the one-cell stage [7].

Another possibility is that the acquisition of capacity for transcription after the 8-cell to 16-cell stage allows the

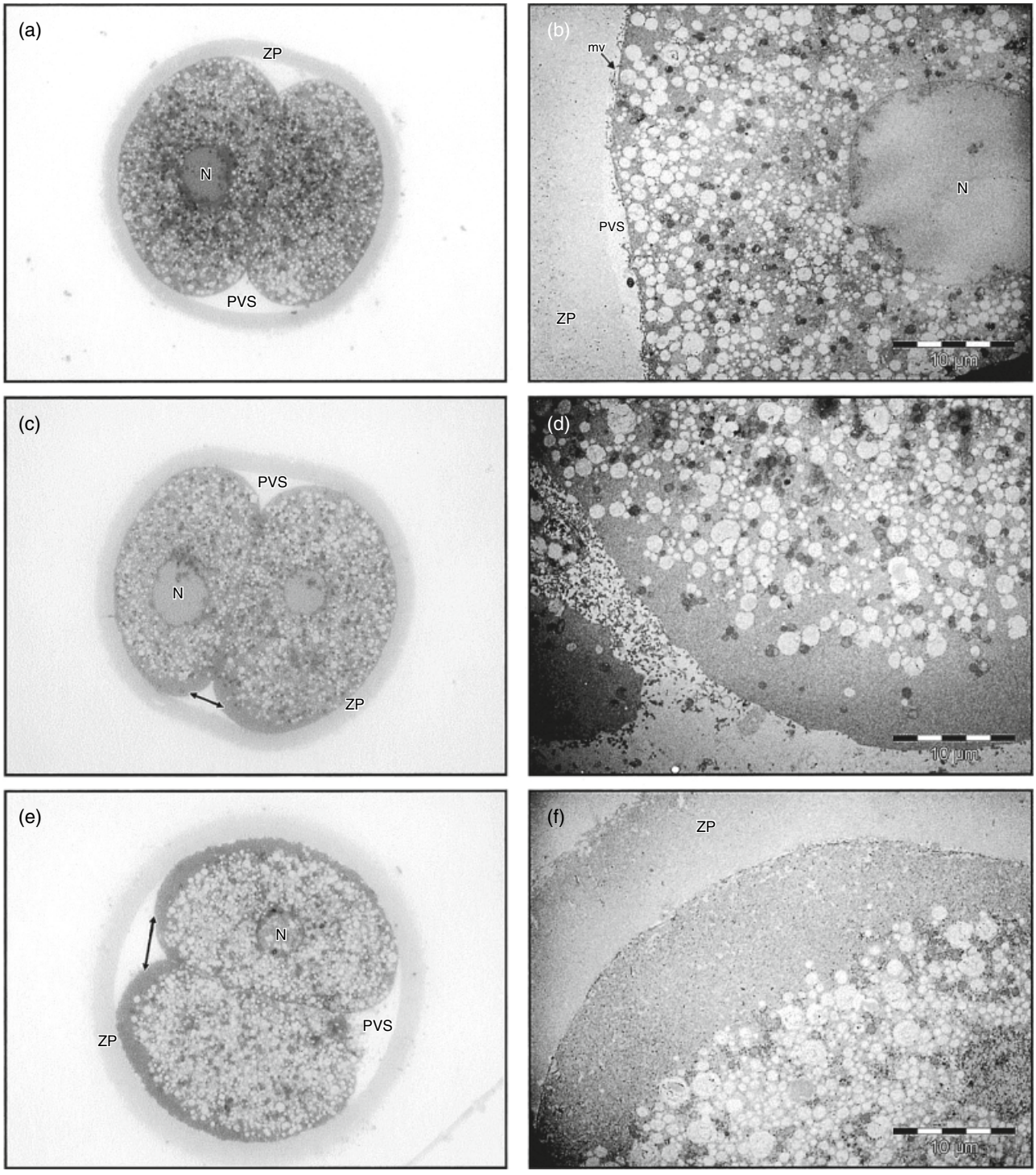


Figure 67.7 Effects of heat shock (41 °C for 6 hours) on the ultrastructure of two-cell bovine embryo produced *in vivo*. Panels (a) and (c) represent images taken using light microscopy after staining with toluidine blue O, while panels (b) and (d) represent images from transmission electron microscopy. Embryos cultured at 38.5 °C are shown in panels (a) and (b), while heat-shocked embryos are shown in panels (c) and (d). Arrows point to an area of the cytoplasm devoid of organelles. ZP, Zona pellucida; PVS, perivitelline space; N, nucleus; MT, mitochondrion; Ly, lysosome. *Source:* Images are reproduced from *Biology of Reproduction* [79] with permission.

embryo to engage a wider range of stress responses to stabilize cellular function during heat shock. Prior to genomic activation, heat-shock-induced transcription of the heat shock protein gene, *HSPA1A*, is not possible [94], although steady-state amounts of mRNA for several stress proteins are higher in the one-cell or two-cell embryo than the morula, including *HSPA1A* [7, 93], probably because transcripts are inherited from the oocyte. Heat shock can also induce synthesis of heat shock protein 70 as early as the two-cell stage [95, 96], possibly through events regulated post-transcriptionally [94].

Other cell stress survival mechanisms may be developmentally regulated. There is, for example, a change in the balance between ROS generation and antioxidant protection as the embryo advances in development. Heat shock caused increased free radical production at day 0 and 2 of development but not at day 4 and 6 [8]. In addition, the cytoplasmic antioxidant glutathione is at its lowest during two- to eight-cell stages [97].

Another developmentally regulated mechanism that protects embryos from heat shock is the capacity for apoptosis. It is not until after embryonic genome activation that proapoptotic signals can activate the apoptosis response; the process is inhibited in two-cell embryos because of mitochondrial resistance to depolarization [98] and inaccessibility of caspase-activated DNAases to the intranucleosomal regions of DNA [99]. Exposure of the morula to 41 °C for nine hours results in about 10–25% of cells becoming apoptotic [100, 101]. Prevention of apoptosis exacerbates the deleterious effects of heat shock on development [100, 101], probably by eliminating those cells most damaged by heat shock.

Heat Stress and Pregnancy Loss

Results are conflicting as to whether late embryonic and fetal loss is affected by heat stress. A controlled experiment to test this hypothesis has not been carried out. Epidemiological analyses indicated an association between warm weather and increased pregnancy loss between day 35–45 and 90 of gestation for cows in Spain [102]. In another study from Spain, increased number of warm events from day 11–20 or 0–40 of gestation was associated with pregnancy loss between day 28–34 and 80–86 of gestation [103]. In contrast, there was no relationship between occurrence of heat stress before or after insemination and pregnancy loss between day 31 and 45 of pregnancy in California [104]. Similarly, there was an absence of seasonal variation in pregnancy loss between day 40–50 and 70–80 or between 70 and 80 and term among dairy cattle in Florida [105].

Physiological Approaches for Improving Pregnancy Success During Heat Stress

At least in high-producing dairy cows, alterations in animal housing to reduce the magnitude of heat stress are important for maximizing reproduction during warm periods of the year. The level of fertility in Israeli dairy farms in the summer, for example, is related to the magnitude of cooling that cows receive (Figure 67.8) [106]. Cooling cows can also increase detection of estrus [4, 108].

To be effective for improving fertility, cooling cannot be limited to a few critical days around ovulation because of the large window of time in which the oocyte is susceptible to heat stress [39]. Short-term cooling around the period of ovulation, fertilization, and early embryonic development causes only slight improvements in pregnancy/AI [4, 108].

Fertility can be depressed in the summer even when cows are housed in facilities with extensive cooling [106, 109]. Consequently, depending on the magnitude of heat stress and the effectiveness of cooling systems, achieving optimal fertility during warm weather depends upon regulating the physiology of the cow to overcome the effects of heat stress on estrous behavior and fertility. In this part of the chapter, some possible strategies for managing reproductive processes during heat stress will be outlined.

Timed Artificial Insemination

Introduction of hormonal protocols to program the timing of ovulation and allow for fixed-time insemination has meant that the effects of heat stress on estrus detection can

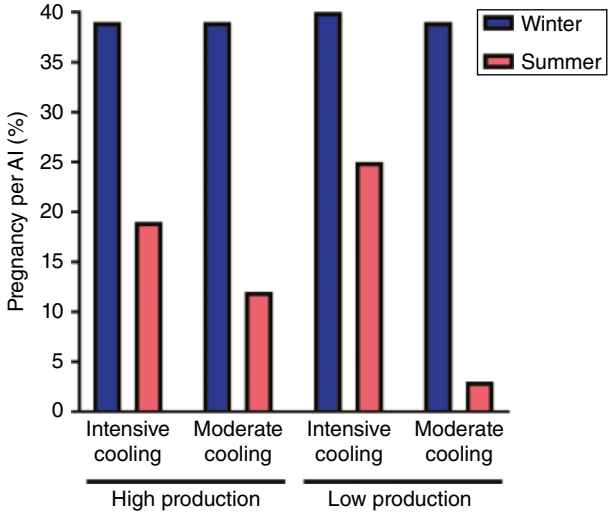


Figure 67.8 Effect of level of milk production (high vs low) and the degree of cooling that cows receive (intensive vs moderate) on fertility of dairy herds surveyed in Israel. *Source:* [107].

be eliminated. Accordingly, use of timed AI protocols during heat stress can increase the 21-day pregnancy rate compared to programs based on spontaneous [110] or prostaglandin-induced estrus [111, 112].

Effectiveness of timed AI protocols may be reduced somewhat during heat stress because there are indications that GnRH is less likely to induce ovulation during heat stress than at cool periods of the year [30]. This consequence of heat stress can be reduced by using more active analogs of GnRH such as dephereline [113]. In addition, effects of heat stress on the oocyte and embryo limit the expected improvement in 21-day pregnancy rate. In several studies, incorporation of timed AI caused no increase in pregnancy/AI [110, 111] or the increase was small [112].

However, new timed AI protocols are designed to increase fertility [114] and there is literature indicating that the relative differences in timed AI protocols for achieving high 21-day pregnancy rates are greater during heat stress. For example, a timed AI protocol involving estradiol cypionate one day before insemination improved pregnancy/AI compared to a protocol utilizing GnRH at insemination when cows experienced two or more days of hyperthermia (rectal temperature > 39.0 °C) but not when cows experienced zero or one day of hyperthermia [115]. In another experiment, the seasonal depression in pregnancy/AI after spontaneous estrus or a timed AI protocol involving insertion of a progesterone releasing intravaginal device (PRID) for nine days (followed by equine chorionic gonadotropin at device removal, GnRH 48 hours later, and AI 12 hours after GnRH) was not observed when the timed AI protocol was modified so that the PRID was inserted for five days only [116].

Embryo Transfer to Bypass Effects of Heat Stress on the Oocyte and Embryo

As illustrated in Figure 67.9, embryo transfer can eliminate much or all of the seasonal variation in fertility caused by heat stress [117–123]. The basis for its effectiveness is the circumvention of effects of heat stress on oocyte competence, fertilization failure, and embryonic death. By the blastocyst stage of development, when an embryo is typically transferred into a recipient female, the embryo has acquired substantial resistance to the adverse effects of elevated temperature [6–8, 77, 78]. Thus, the pregnancy success after embryo transfer during heat stress is much higher than following insemination. This is the case whether embryos were produced by superovulation [122–125] or *in vitro* production techniques [126–128]. The *in vitro* produced embryo can experience poor cryosurvival [129], so benefits of embryo transfer during heat stress are sometimes reduced [128] or eliminated [125] if cryopreserved embryos produced *in vitro* are transferred.

Embryo transfer can be used in conjunction with the same ovulation synchronization programs for timed AI [21, 126–128] so that actions of heat stress on detection of estrus can be eliminated for embryo transfer as well as for AI.

Progesterone Supplementation to Improve Embryo Survival

Hormonal treatments designed to increase circulating concentrations of progesterone after breeding can increase cow fertility [130]. This may also be the case during heat stress. Treatment of lactating dairy cows with human chorionic gonadotropin at day 5 or 6 after AI did not improve fertility in one experiment [131] but increased pregnancy/AI in cows regardless of parity [132] or for primiparous cows only [133]. Insertion of a progesterone-containing intravaginal device on day 5 ± 1 for 13 days increased pregnancy/AI when cows were in low body condition only [134]. Similarly, treatment with GnRH at AI or AI and day 12 after AI also increased pregnancy/AI [135].

Antioxidants

Exposure to elevated temperature increases ROS production by oocytes [63, 65] and embryos [8, 81]. Moreover, heat stress can increase markers of oxidative stress in the blood [136, 137]. Mixed results have been obtained from the few experiments evaluating whether provision of supplemental antioxidants can improve fertility during the summer. There was no improvement in fertility when cows were injected with vitamin E at insemination [4], given multiple injections of vitamin E and selenium before and after calving [85], or administered injections of β-carotene at −6, −3, and 0 days relative to insemination [138]. In contrast, feeding supplemental β-carotene for at least 90 days beginning at ~15 days after calving increased the proportion of cows that were pregnant at 120 days postpartum [110]. In addition, treatment of cows with melatonin implants in the summer reduced days open [139]. Interpretation of the melatonin study is difficult because melatonin has many biological actions, including as an antioxidant [65, 140] but also as an important mediator of photoperiodic regulation [141].

Follicle Removal to Eliminate Damaged Follicles After Heat Stress

Work in Israel has shown that inducing removal of follicles from the ovary in the autumn can hasten the restoration of fertility following heat stress. Beneficial effects on oocyte quality have been observed by turning over the follicular pool using follicular aspiration [50] or injection with either follicle stimulating hormone [142] or somatotropin [142].

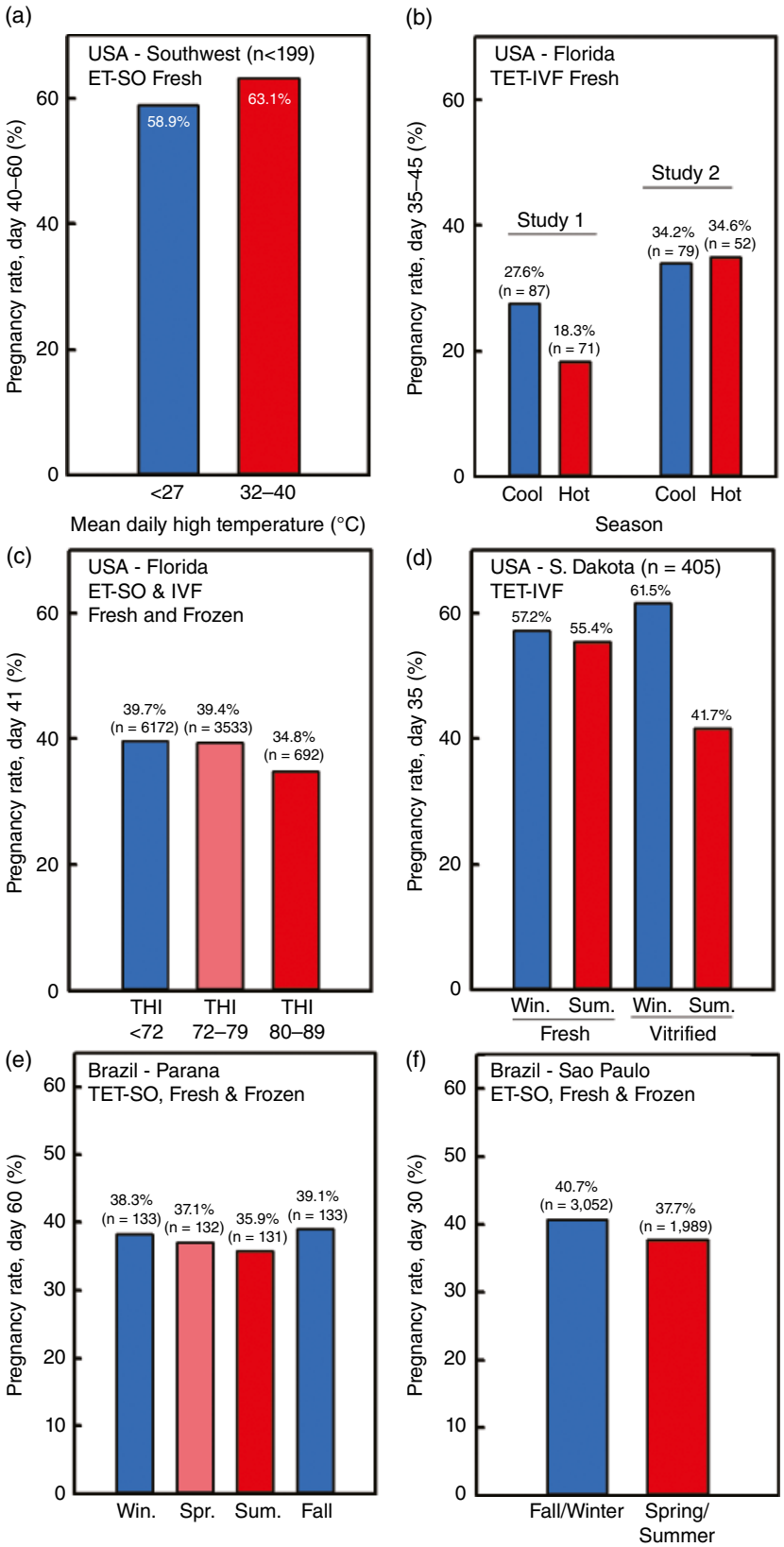


Figure 67.9 Pregnancy success after embryo transfer in cool and hot weather in lactating dairy cows. Note that in most studies there was little variation in percentage of females pregnant between cool and hot periods. ET, Embryo transfer; IVF, *in vitro* fertilized; S. Dakota, South Dakota; Spr., spring; SO, superovulation; Sum., summer; TET, timed embryo transfer; THI, temperature–humidity index; Win., winter. *Source:* [107].

Such treatments would not be expected to be effective in the summer because follicles emerging into the growing pool would also be damaged by heat stress. Consistent with this idea was the observation that treatment of lactating cows in the summer and autumn with three rounds of a GnRH and $PGF_{2\alpha}$ protocol to hasten removal of follicles did not improve pregnancy rate at first AI in multiparous cows, although it tended to improve pregnancy rate in primiparous cows [143]. Perhaps greater responses would have been seen if the study was limited to the late summer or autumn.

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The Reproductive Tract Microbiome: Implications for Fetal and Neonatal Health

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Introduction

A new and expanding area of research in both human medicine and animal science is evaluating microbial diversity within different organ systems. In humans, the gut microbiome has been referred to as a supporting organ because of the numerous associations between the gut microbiome and overall host health [1, 2]. Additionally, negative impacts on host microbiomes throughout the body can have detrimental effects on overall health [3]. Therefore reproductive physiologists have begun investigating the roles that microorganisms could play in fertility and reproductive performance. Current research into the microbiome of the reproductive tract is promising but incomplete. The objective of this chapter is to highlight current impactful studies evaluating the microbiome of the female reproductive tract and provide direction for future research.

Microbiome Research Methodology

Bacteria, both pathogenic and commensal, that directly inhabit and co-exist in any environment are referred to as the bacterial microbiota of that environment's microbiome [4]. Researchers heavily agree that commensal bacteria are crucial for the maintenance of homeostasis in both human and livestock species [5, 6]. It has been determined that each body site harbors a unique microbial community that can differ completely from one site to another [7]. Therefore researchers have migrated from evaluating the entire microbiome of a host to instead evaluating specific microbiomes within the host's physiological systems [8]. Throughout the development of microbiology research, specific terminology, methodology, and data presentation have been adapted. Thus, before current literature can be discussed in depth, an overview of methods and data analyses unique to microbial research must be presented.

Firstly, obtaining a pure uncontaminated sample requires stringent methods dependent on the specific microbiota being sampled. The results of a study can be heavily impacted by sample collection methods. For example, the gut microbiota can easily be evaluated by collecting a stool sample, but sampling the microbiota of a specific organ would require sterile invasive procedures. Additionally, direct collection methods, such as stool collections, yield an extremely microbial dense sample with minimal contamination risk [7]. Conversely, utilizing swabs or lavages to collect samples proves more challenging to obtain samples without contamination and is less studied regarding protocol consistency and accuracy [9]. Typically, swabs are the least invasive but retrieve the smallest microbial biomass, whereas biopsies are more invasive but collect the most microbial biomass [7, 10]. Therefore, when determining how to sample, the researcher must consider sampling site, contamination risk, and how invasive their sampling procedure is.

After sample collection, the most common analysis used for evaluating microbiota composition is gene sequencing utilizing the 16S rRNA gene [11]. This method is not perfect but has been proven an objective, accurate, and reliable method for bacterial identification [12]. A highly conserved component of the transcription machinery of a microorganism's DNA is the 16S rRNA gene [13]. Therefore 16S rRNA is a great target gene for sequencing DNA in samples that contain thousands of different microbial species [13]. Additionally, the 16S rRNA gene is large enough (1500 base pairs) for information purposes, its function has not changed over time, and it is present in almost all bacteria and archaea, further attesting its use for studying microorganisms [14]. The 16S rRNA gene has both variable and conserved regions, and the variable regions differ between

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

specific microorganisms; this allows for differentiation between microbial species [15].

The methodology for the bioinformatic analysis of the microbiota is referred to as a pipeline. A pipeline utilizes bioinformatic software programs and laboratory kits which can change dependent on individual preference and the goal of the study, but the general steps taken within analysis are the same.

The first step within microbial analysis is DNA extraction from collected samples, the extracted DNA then undergoes quality/quantity check, next the universal 16S rRNA primers are used to amplify all bacterial DNA via polymerase chain reaction (PCR), then the 16S rRNA is sequenced from the bacterial DNA, and finally similar 16S gene sequences are clustered into operational taxonomic units (OTU) and identified [16]. OTU are groups of similar 16S rRNA reference sequences; typically, they are grouped at a certain percentage of similarity, such as 95 or 98% [17]. After clustering into OTU, a reference database, which has current information about what 16S gene sequences match specific bacterial taxonomy, is utilized to identify the species each OTU represents [18]. It is important to mention that not all sequences identified as an OTU will have a taxonomic match within the database. This occurs because that specific sequence has yet to be identified and assigned taxonomy. After the bacterial community has been characterized, statistical analysis for significant differences is the next step [16].

Most researchers present data analyzing the alpha and beta diversity of samples, as this is a quantitative analysis of comparable differences within and between samples, respectively. First, alpha diversity is the diversity within a sample that specifies species richness and evenness [19]. Alpha diversity is commonly measured via the Shannon diversity index, a statistical method that accounts for species abundance/richness and evenness within a sample [20]. Richness, or abundance, refers to the number of observed species within an individual sample. Evenness is the relative abundance of species within a sample [21]. Further explanation of alpha diversity is illustrated in Figure 68.1. Beta diversity is the extent of change in species composition in different samples [22]. The statistical methods to analyze differences in beta diversity are dependent on researchers, and commonly researchers utilize a non-permutational index (such as Bray-Curtis) accompanied with a permutational multivariate analysis of variance (PERMANOVA). Normally beta diversity is visualized using a principal component analysis which allows a visualization of the similarities between each sample [23]. This statistical graph allows readers to visualize the beta diversity between samples.

The aforementioned methods have provided insight about microbial composition throughout physiological systems in many species and the inner workings of microbial presence on overall health. Although many species and physiological systems have been analyzed, there is still much room for growth and the expanding of knowledge in microbiota research. A recent trend in microbiota research is to evaluate the microbiota of the reproductive tract and its impacts on fertility and reproductive performance.

The Microbiota of the Human Reproductive Tract

To date, the most in-depth research characterizing reproductive tract microbiotas, including the vagina, uterus, placenta, and amniotic fluid, has been performed in humans. Specifically, researchers are focusing on first determining if a microbiota exists, then characterizing the microbiotas located within the reproductive tract, and lastly, determining how the microbiota can affect fertility.

The vaginal microbiota and physiological environment of the vaginal tract was characterized by Ravel et al. [24]. In this study, the vaginal bacterial communities of reproductive age women (n = 396) were analyzed using the 16S rRNA gene. Subjects were of different ethnic and racial backgrounds (Caucasian, African-American, Hispanic, and Asian) and all were currently living in the USA. Results found that the majority of bacteria within the subjects' vaginal tracts had the common physiological ability to produce lactic acid. It was concluded that Caucasian and Asian women had different vaginal microbiotas than African-American and Hispanic women. Results also showed that the vaginal tracts of Caucasian and Asian women were primarily dominated by *Lactobacillus* species, but the vaginal tracts of African-American and Hispanic women had fewer *Lactobacillus* species. Previously, it was proposed that the production of lactic acid by *Lactobacillus* species in the women's vaginal microbiota was crucial for vaginal health [25]. However, Ravel et al. [24] dispute this claim and state that many factors including ethnicity and environment could contribute to the composition of the vaginal microbiota. Lastly, authors found that the pH of Caucasian and Asian women was significantly different from African-American and Hispanic women (4.2 compared to 5.0). Therefore the two groups clearly had different amounts of lactic acid production in the vagina.

Lactic acid facilitates an acidic vaginal environment, which can prevent the growth and attachment of potential pathogens [26]. Ronnqvist et al. [27] evaluated the role of *Lactobacilli* species in the vaginal tract and their relationship to other genital microbes and vaginal pH. In 191 women evaluated, the overall conclusion was that *Lactobacilli* species contribute to a low vaginal pH, which

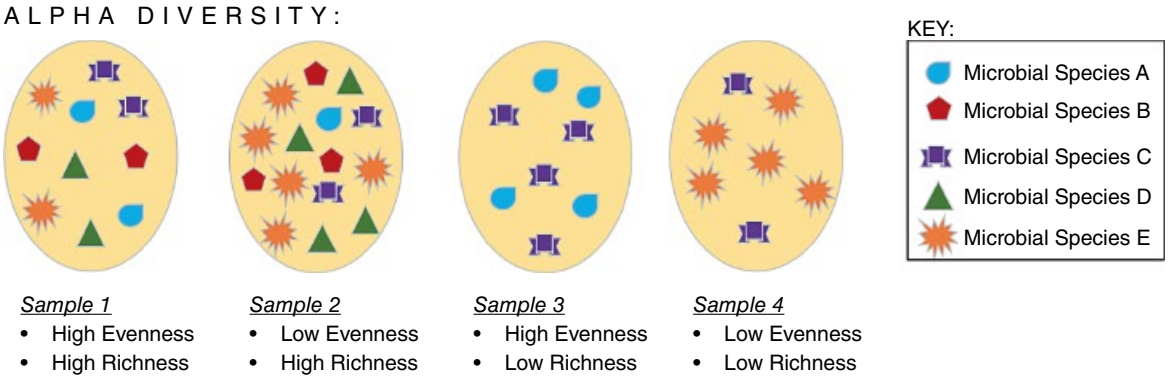


Figure 68.1 Alpha diversity is the microbial diversity within a sample. Alpha diversity is measured in evenness and richness. High evenness is when the population proportions are similar within the samples. For example, in samples 1 and 3 there are two of each microbial species and three of each microbial species, respectively. Therefore the microbial proportions are even within these samples. High richness is when there is a high number of microbial species within a sample. For example, in samples 1 and 2 all microbial species are represented, so these samples have high richness compared to samples 3 and 4 that only have two microbial species represented. See text for citations.

has a negative impact on the growth of Group B Streptococcus. Group B Streptococcus is associated with preterm birth, fetal injury, and neonatal mortality in newborns; recently, it was discovered that this pathogen also has the ability to infect fetuses in utero [28]. Therefore it can be inferred that an acidic vaginal environment is beneficial in women for preventing growth of pathogenic bacteria, but, as mentioned in Ravel et al. [24], the physiologically normal pH and bacteria that induce the acidic environment are different among ethnic groups. Therefore further characterization of the normal vaginal microbiota in different demographics of women is necessary.

After the original characterization studies, vaginal microbiota researchers shifted to determining if a microbiota is present within the uterus. Similar to animal species, obtaining sterile samples from the uterus in women is difficult due to anatomical barriers such as the vaginal tract and cervix. Data showing microbial populations in the uterus have been published and presented in the literature, but many researchers are still skeptical of the presence of a uterine microbiota and attribute current findings to contamination [29]. Additionally, if a microbiota exists, it is debatable if it has positive or negative effects on reproductive performance, fertility, or pregnancy establishment.

Within the uterus, the most abundant phyla reported include Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria [30, 31]. This sampling pool included patients with both healthy and unhealthy reproductive tracts; therefore attributing microbial composition to uterine health appears difficult and unreliable at this time. Additionally, *Lactobacillus*, from the Firmicutes phyla, is the most abundant genus of the uterus in the aforementioned studies and others [32–34]. Recall that the colonization of the vaginal tract with *Lactobacillus* species led to decreased vaginal pH to prevent growth of pathogens, but within the uterus its role is up for debate.

Fang et al. [35] reported that women with endometrial polyps and chronic endometriosis had a higher relative abundance of *Lactobacillus* species in the uterus than healthy women, concluding that *Lactobacillus* has a negative effect on reproductive performance. However, Moreno et al. [36] reported that high levels of *Lactobacillus* species in the uterus increased success rates in women undergoing *in vitro;* fertility treatments and embryo implantation success. Interestingly, there are regular findings of *Lactobacillus* species within the uterine tract, but an obvious function or physiological role within the uterus is unknown. It is plausible that vaginal contamination of the sample occurred, and the research being reported is not actually to do with the uterine microbiota but a result of contamination. Therefore more in-depth research with multiple contamination checks needs to be implemented before a uterine microbiota can be confirmed.

Finally, researchers have analyzed tissue from the placenta and amniotic fluid during pregnancy to detect the presence of a microbial community. If a microbiota does exist, there could be major implications surrounding fetal microbial inoculation, fetal development, and fetal health both in utero and after birth [37].

Researchers studying the placenta and amnion in humans have found microbial populations present, but there is still much debate on whether it is colonization or contamination. In a study by Stinson et al. [38], amniotic fluid and fetal meconium were collected from women undergoing non-emergency cesarean sections (CSs) ($n = 50$). Each sample was characterized via 16S rRNA gene sequencing. They found that all meconium samples contained a high number of reads that matched *Pelomonas*

puraquae, and amniotic fluid samples had a low number of reads and microbial diversity with only skin commensals being present. Within their discussion, the authors acknowledged the possibility of contamination of meconium samples with *P. puraquae* within the laboratory, but largely concluded that the overall presence could not solely be due to external contaminants [38]. The authors attributed the presence of *P. puraquae* only in the meconium to the bacteria's ability to survive in the harsh environmental composition of meconium, whereas skin commensals were mostly found in the amniotic fluid because amniotic fluid typically contains high amounts of sloughed fetal skin cells [39].

Conversely, Lim et al. [40] found that all microbes present within the amniotic fluid were due to sample contamination either in the birthing suite, laboratory, or transport. This study was in agreement with previous literature regarding the low bacterial biomass in amniotic fluid samples [38, 40]. Due to the low bacterial biomass, current researchers in this field are still determining the best way to process and analyze these samples. Recent discrepancies with the materials and methods of Lim et al. [40] have been published in the literature [41]. Overall, concrete evidence supporting presence or absence of a microbiota in the amniotic fluid has yet to be presented in the literature.

Lastly, similar to the amniotic fluid, there is a debate in the literature of the existence of a placental microbiota. Aagaard et al. [42] found that the placenta contained a unique microbiota when sampling 320 women. The placenta was primarily inoculated with commensal bacteria. This study included samples from 320 women with both healthy and preterm births, and it was concluded that all placentas sampled had a low microbial biomass. Although interesting, the Aagaard et al. [42] study had some limitations that severely compromised the integrity of the paper in terms of validity; due to no contamination checks being reported or analyzed, there is no possible way to know if the microbial communities reported in this study were due to contamination or colonization.

The most impactful research evaluating the placental microbial population was done by Goffau et al. [43] which refutes claims from previous researchers that the placenta contains a microbiota. In this study, placentas (n = 537) from women that had vaginal deliveries, prelabor CS, and CS after labor onset were evaluated. Rigorous contamination evaluations were performed to determine if contamination occurred in either the delivery room or laboratory. Results showed that all microbiota present on the placenta samples were from contamination. However, native pathogens, specifically Group B Streptococcus, were discovered on the placenta samples. Therefore the authors concluded that while the placenta does not have a resident microbiota, it can harbor pathogens that can have fatal consequences to infant's post-partum.

Interestingly, through their evaluation of contamination, the authors also found that the placenta was naturally inoculated with vaginal microbiota during natural birth and CS after labor onset only. This suggests the ascension of vaginal microbiota through the cervix during labor to inoculate the placenta and fetus. Ultimately, if valid, the vaginal microbiota would be the first contact the fetus has with microorganisms, suggesting that the composition of the vaginal microbiota could be associated with the cause or prevention of neonatal diseases. While more research is needed to verify this study, it can be speculated that the vaginal microbiota is extremely important in the reproductive and overall health of women and their neonates.

Clearly, the research and evaluation of the role and presence of microbiota in the reproductive tract of humans is incomplete; however, previous research in humans can assist animal scientists in designing studies to evaluate the microbiota of the reproductive tract in livestock species.

The Microbiota of the Bovine Reproductive Tract

Literature covering the characterization and implication of the microbiota within the reproductive tract of bovids is scarce. Unfortunately, most of the literature in bovids and livestock species available regarding the reproductive tract microbiota has severe limitations, such as no contamination checks or fewer numbers of animals. Similar to humans, the colonization of the reproductive tract with microbiota could reveal linkages between microbial communities and fertility, reproductive tract health, and overall animal health. To date, the most researched microbiota of the reproductive tract is the vaginal microbiota and its role in reproductive performance.

Swartz et al. [44] found that the vaginal environment and microbial community composition of humans and bovids differ. This study utilized crossbred cows (n = 20) and compared the microbial data results to previous literature characterizing women's vaginal microbiota. These researchers found that bovids' vaginal tracts have low levels of *Lactobacilli* and near-neutral pH compared to humans. Due to the change in pH, it can be expected that the commensal vaginal bacteria within bovids produce less lactic acid and require a near-neutral pH for optimal growth. This was further demonstrated in the characterization of the bovine vaginal microbiota, with the three most abundant phyla being Bacteroidetes, Fusobacterium, and Proteobacteria [44]. Therefore previous literature associating the human vaginal

microbiota to fertility may not be directly applicable to bovids [45].

A study by Nascimento et al. [46] evaluated the vaginal microbiota in four subsets of bovids, each unique in their reproductive status. The Nelore cattle breed was utilized with pregnant cows (n = 5), non-pregnant cows (n = 5), pregnant heifers (n = 5), and non-pregnant heifers (n = 5) included in the study. In agreeance with Swartz et al. [44], the most abundant phyla in all animals were Firmicutes, Bacteroidetes, and Proteobacteria. Within this study, they found that great microbial variability exists among animals, and no statistical differences were found as a result. Additionally, this study was the first to mention the potential role of reproductive hormones influencing the composition of the vaginal microbiota, but no evidence was provided. Perhaps the most interesting finding from the study was the lack of obligate anaerobes in non-pregnant heifers' vaginal microbiotas. Typically, obligate anaerobes are the last bacterial community to colonize a specific environment [47]. Authors attributed this finding to the potential of an underdeveloped vaginal microbiota in heifers which could lead to decreased fertility in these animals [46].

Estrus synchronization protocols in cattle could potentially impact the vaginal microbiota, more specifically protocols that utilize vaginal inserts to deliver hormones. Moreno et al. [48] investigated the difference in vaginal microbial communities post-progesterone releasing intravaginal devices (PRID) in cows and heifers. Post-PRID removal the animals were classified as having either vaginitis or no vaginitis (healthy); this was determined by counting polymorphonuclear neutrophils in vaginal cytobrush samples. They found that cows and heifers with vaginitis had higher Proteobacteria, a more uniform microbiota, and an increased prevalence of opportunistic pathogens within their vaginal microbiota. Overall, vaginitis caused by the use of PRIDs could increase the prevalence of bacteria in the vaginal microbiota, which could act as uterine pathogens and negatively impact fertility.

In addition to maternal health and fertility, the vaginal microbiota could have impacts on the dam's offspring. Lima et al. [49] sampled the vaginal and fecal microbiota of Holstein cows (n = 81) and compared it to the fecal and upper respiratory tract (URT) microbiota of their calf (n = 81), at 3, 14, and 35 days old, to evaluate if the maternal microbiota could influence calf health. They found that the URT microbiota of calves and the dams' vaginal microbiota were highly similar (63% overlap with 253 shared OTU) regardless of days of life. *Mannheimia* and *Moraxella*, bacteria involved in bovine respiratory disease in calves, were some of the most prevalent shared between dam and calf, suggesting that the health of a calf could be affected by mother-to-offspring transmission during parturition.

Messman et al. [50] evaluated the role of estradiol and pregnancy status on bacterial community composition of the vaginal microbiota. Researchers sampled Brangus heifers (n = 78) at timed artificial insemination (TAI) to determine if there were differences in bacterial composition between pregnant and non-pregnant heifers or heifers with divergent estradiol concentrations. It was determined that the bacterial composition of the vaginal microbiota was not changed by estradiol concentrations at time of artificial insemination. Additionally, heifers that became pregnant versus non-pregnant heifers did not have a different vaginal microbiota at time of artificial insemination.

A study by Ault et al. [51] investigated the role of progesterone concentrations altering the vaginal and uterine microbiota and compared the vaginal and uterine microbiotas of pregnant and non-pregnant post-partum cows prior to TAI. Samples were collected from pregnant (n = 10) and non-pregnant (n = 10) cows at three time points (day 21, day 9, and day 2 prior to TAI) from the vagina and uterus via lavage; blood serum was also collected at the aforementioned time points for progesterone concentration analysis. The estrus synchronization protocol utilized in this study did not incorporate a vaginal insert (CIDR/PRID).

There were no differences in the alpha diversity of vaginal microbiota species abundance at any sample date prior to TAI, but the authors found that OTU significantly decreased in the uterus over time [51]. Ault et al. [51] utilized a principal coordinate analysis to visualize beta diversity within the samples. Results showed significant clustering at day 2 in the uterus ($P = 0.005$) and in the vagina at day 21 ($P = 0.002$) between pregnant and non-pregnant cows. The authors proposed the differences in the uterus at day 2 could be affecting pregnancy establishment in these animals. They also found that the composition of the uterine and vaginal microbiotas varied in beta diversity dependent on day of collection. Lastly, the authors found no direct relationship to progesterone concentrations and the vaginal and uterine microbiotas.

Overall, Ault et al. [51] agrees with previous literature stating that there is high variation and diversity within the microbiotas of the bovine reproductive tract [46, 44]. The authors suggested that close clustering of non-pregnant animals at day 2 prior to TAI is due to a group of closely related bacteria within the uterus that can prevent or decrease the likelihood of pregnancy establishment. Limitations from this study are the small sample size and lack of contamination checks.

The study by Ault et al. [51] is one of the few studies within bovids evaluating the uterine microbiota in live animals before parturition. Moore et al. [52] sampled the entire reproductive tract of virgin heifers and pregnant

cows. This study aimed to establish that the uteri of pregnant cows and virgin heifers possessed a resident microbiota. Researchers collected the endometrial samples of 10 virgin dairy heifers via biopsies, and collected amniotic fluid, placentomes, intercotyledonary placenta, cervical lumen, and vaginal tissue from five pregnant dairy cows after slaughter. All samples generated a microbiota, leading researchers to conclude that both pregnant and virgin uteri contained a resident microbiota. They found the three most abundant phyla in both the pregnant and virgin uteri were Firmicutes, Bacteroidetes, and Proteobacteria. These results led them to conclude that the uterine microbiota is established before females reach reproductive maturity. Therefore the uterus is not a sterile environment at the establishment of pregnancy and could potentially inoculate the calf in utero [52]. However, no contamination checks were mentioned in the publication and tracts of pregnant animals were collected on a processing floor where the possibility of contamination is extremely high.

The most literature evaluating bovine uterine bacterial communities has been studied in post-partum cows to evaluate the microbial abundance/composition and the risk of developing uterine disease, such as metritis. A study by Wang et al. [53] focused on characterizing the uterine microbiota of post-partum dairy cows with clinical (CE) and subclinical (SE) endometritis. Researchers collected uterine flush samples from 13 healthy, 5 SE, and 9 CE cows at 30 days post-partum. All cows had a similar microbiota with 293 of 445 shared OTUs. Clinical endometritis was characterized by an increased abundance of *Fusobacterium* and presence of *Trueperella* and *Peptoniphilus*. SE cows had no uterine pathogens but an increased abundance of *Lactococcus* and *Acinetobacter*. This study concluded that healthy, SE, and CE cows share a similar microbiota; however, the competitive and cooperative interactions within the microbiota still need to be established to determine how to combat uterine pathogens and promote a healthy uterine microbiota.

Santos et al. [54] found that the phylum Fusobacteria was affiliated with metritic post-partum cows, but in this study, no differences were found between healthy and metritic cows. Interestingly, Jeon et al. [55] evaluated the possibility of blood introducing pathogens into the uterus post-partum. They compared bacterial communities from blood, feces, and uterine samples at day 0 and day 2 post-partum and vaginal samples at seven days prior to calving. Results of the principal coordinate analysis showed distinct clustering of blood and fecal bacterial communities, but more scattered clustering of uterine and vaginal communities indicating greater variability [55]. Additionally, major uterine pathogens were detected in blood samples and there was a strong and significant interaction of uterine pathogens and the blood microbiota. The authors concluded that in addition to fecal and vaginal microbial contaminates, uterine bleeding post-partum can lead to the introduction of pathogens to the uterus.

Researchers who evaluate post-partum uterine microbiota generally assume that any microbial populations present are contaminates that contribute to decreased reproductive performance. This is in stark contrast to the Ault et al. [51] and Moore et al. [52] studies that proposed that the presence of a commensal uterine microbiota could facilitate pregnancy. Ultimately, further research will be needed to determine if a uterine microbiota exists in bovids; these studies need to implement contamination checks similar to human studies and be cognizant of contamination throughout the entire analytical process.

Implications of the Vaginal Microbiota in Compromised Pregnancy

Literature evaluating the role of the reproductive tract microbiota in bovine pregnancies that are compromised is sparse. The negative consequence of compromised pregnancy is intra-uterine growth restriction (IUGR) of the conceptus that has negative effects both in utero and after birth. There are many mechanisms that cause IUGR, but one of the most notable and prevalent in the beef cattle industry is nutrient restriction. Therefore researchers have spent decades studying nutrient restriction, IUGR pregnancies, and potential supplements to mediate the effects of both. Despite this breadth of knowledge, the implications of the vaginal microbiota in compromised pregnancies are unknown.

IUGR is defined as the retardation of growth and development of a mammalian conceptus during pregnancy [56]. The offspring of dams that experience nutrient restriction, especially during the third trimester when the majority of fetal growth occurs, are extremely susceptible to IUGR [57]. Primarily, IUGR is caused by the limitation of uterine capacity, translating to a decrease in nutrient flux to the fetoplacental–maternal unit [58]. This results in diminished nutrient availability to the fetus. The limited nutrients available to the fetus are shunted to primary organs, the brain, and necessary physiological systems that are critical for life [59]. As a result, IUGR calves characteristically have a smaller birth weight and larger head to body ratio, and are metabolically altered throughout their life [60]. Additionally, these calves tend to be weak, struggle earlier on in life, have a higher mortality rate, and can be an economic burden to producers [61]. Therefore researchers have begun to focus on mediating the effects of

nutrient restriction during pregnancy to counteract the negative effects of IUGR on calf performance.

Although several animal models have been developed to unravel the relationship between placental capacity and IUGR, there has been limited research evaluating how the reproductive tract microbiota is affected in IUGR pregnancies. In mice, pups experiencing IUGR had alterations of the intestinal microbiota [62]. In piglets experiencing IUGR, researchers found an unbalanced gut microbiota, impaired small intestine structure, and abnormal inflammatory and metabolic profiles 12 hours after birth [63]. A model for how microbial communities could influence IUGR pregnancies in women was described by Bardos et al. [64]. They postulated that dysbiosis throughout the maternal microbiota caused by pathogens can result in systemic inflammation, including inflammation of uterine and placental tissues [64]. The inflammatory response in these tissues could lead to decreased surface area for nutrient exchange, resulting in an IUGR fetus. Overall, these studies indicate that if the maternal microbiota is in a state of dysbiosis during pregnancy, systemic inflammation could result. Furthermore, during parturition the fetus is inoculated with the dam's microbiota; thus the newborn is exposed to a potentially pathogenic microbiota which results in alterations of fetal metabolic and inflammatory responses.

Houdijk et al. [65] found that during gestation metabolizable protein becomes scarce and nutrient partitioning will prioritize protein transport to the reproductive tract instead of the immune system. Thereby, an animal that is nutrient restricted will also be immunocompromised to a certain extent. This immunocompromised state, accompanied by the already immunosuppressive state of the reproductive tract during gestation [66], provides the perfect situation for the inoculation of the vaginal microbiota with potential pathogens. If the vaginal microbiota does become inoculated with pathogenic bacteria during gestation then localized inflammation in the reproductive tract will be present, the calf will be exposed to the pathogens during parturition, and the potential for a post-partum infection is severely increased. Therefore the implications of the vaginal microbiota in IUGR pregnancies is overwhelming, yet there is a paucity in the literature.

Summary

In conclusion, the study of the microbial communities of the reproductive tract is a new and emerging area of research. Most of the published literature, especially in bovids, has limitations which negatively impacts the validity of the research. However, these studies are important for characterization of the microbiota. More studies will be needed to characterize the reproductive tract microbiota throughout the estrous cycle, pregnancy, post-partum, and any other significant reproductive events. Once characterization is completed, comparison studies can occur and perhaps mechanisms of pathogenesis will be revealed. Ultimately, the long-term goal is to determine if a certain commensal bacteria community within the bovine vaginal tract facilitates the establishment of pregnancy or if the presence of pathogenic bacteria negatively impacts fertility, fetal development, or pregnancy establishment. More research will be needed to reveal the answers to such questions, but this field has promise for furthering knowledge in reproductive physiology and improving current production methods to increase pregnancy rates and economic profitability.

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69

Bovine Abortifacient and Teratogenic Toxins

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Introduction

A toxicant is a material that interferes with normal biological processes and causes adverse effects when it enters the body via ingestion, inhalation, dermal contact, or injection [1]. Reproductive toxicants are those capable of causing infertility, subfertility, early embryonic death, fetal developmental deformities (teratogens), fetal death, abortion, and stillbirths. Reproductive toxicants can cause deleterious effects by acting alone or by interacting with environmental, nutritional, or infectious stressors [2]. The effect of naturally occurring toxicants as well as anthropogenic compounds on reproduction in cattle while often sporadic can have considerable economic impacts [3].

Nitrate-/Nitrite-Accumulating Plants

Ruminants, particularly cattle, are at increased risk for developing acute nitrate/nitrite poisoning from the ingestion of excessive amounts of nitrate-accumulating forages, weeds, and crops. Plants attain nitrogen from the soil in the form of nitrate and nitrite. Excessive nitrogen accumulation may occur in a variety of grasses and weeds under specific environmental and artificial conditions including increased fertilization, drought, hail, frost, herbicide damage, decreased sunlight, increased soil acidity, and in soils deficient in sulfur, phosphorus, and molybdenum. Commonly incriminated nitrate-concentrating plants include cereal grains, forage crops, and common weeds [2, 4, 5]. Nitrate accumulates greatest in the roots and lower portions of the plant including the stalks and stems due to decreased photosynthesis-dependent nitrate reductase activity at these sites [2]. Cattle may also ingest excessive nitrite and nitrate compounds directly from nitrate-based fertilizers (storage of fertilizers in areas accessible to cattle) and from water contaminated with manure or fertilizer runoff [4, 6]. A few of the more commonly implicated nitrate-accumulating weeds are Johnson grass (*Sorghum halepense*), pigweed (*Amaranthus retroflexus*), jimsonweed (*Datura stramonium*), and lamb's quarters (*Chenopodium* spp.) [6, 7]. However, a variety of other plant species have also been implicated in nitrate poisoning [4] (Table 69.1).

Pathogenesis/Mechanism of Action

Normally dietary nitrate is converted to nitrite within the reticulorumen and is slowly metabolized to ammonia. However, when present in levels that overwhelm the microbial organisms' ability to reduce them, nitrate and nitrite are rapidly absorbed into the circulation. Once in the circulation nitrite ions readily interact with hemoglobin molecules of blood, promoting the oxidation of hemoglobin iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state and forming methemoglobin. The methemoglobin molecule is incapable of binding and transporting oxygen molecules. Once sufficient hemoglobin is oxidized to methemoglobin, clinical signs of hypoxia develop including exercise intolerance, dyspnea, muscle tremors, and cyanotic and occasionally brown-colored membranes and blood. Although maternal methemoglobin molecules are incapable of crossing the placenta, nitrite and nitrate readily cross and can induce fetal erythrocyte methemoglobinemia and anoxia. Typically, reproductive repercussions including abortions, stillbirths, and the birth of weak calves have been reported approximately three to seven days or more following maternal toxic exposure. In addition to the effects of methemoglobinemia, nitrite and nitrates act as potent vasoactive compounds resulting in vasodilation and hypotension [2, 8].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Table 69.1 Nitrate-accumulating plants.

Sorghum sudan (*Sorghum vulgare*)
Pigweed (*Amaranthus* spp.)
Button grass (*Dactyloctenium radulans*)
Sugar beet (*Beta vulgaris*)
Corn (*Zea mays*)
Soybean (*Glycine max*)
Pearl millet (*Pennisetum typhoides*)
Flax (*Linum* spp.)
Rye (*Secale cereale*)
Sunflower (*Helianthus annuus*)
Oats (*Avena* spp.)
Lamb's-quarter (*Chenopodium* spp.)
Turnips and rape (*Brassica* spp.)
Canada thistle (*Cirsium arvense*)
Wheat (*Triticum aestivum*)
Goldenrod (*Solidago* spp.)
Nightshades (*Solanum* spp.)
Barley (*Hordeum* spp.)
Sorrel, curly leaf dock (*Rumex*)
Alfalfa (*Medicago sativa*)
Russian thistle (*Salsola kali*)
Johnson grass (*Sorghum halepense*)
Sweet clover (*Melilotus* spp.)
Tall fescue (*Lolium arundinaceum*)
Kochia weed (*Kochia scoparia*)
Timothy grasses (*Phleum* spp.)
Smart weed (*Polygonum* spp.)
Ragweed (*Ambrosia* spp.)
Jimsonweed (*Datura stramonium*)
Fireweed (*Kochia* spp.)
Field bindweed (*Convolvulus arvense*)
Docks (*Rumex*)
Barnyard grass (*Echinochloa crus-galli*)
Cheese weed (*Malva* spp.)

Source: Adapted from [4].

Diagnosis

In adult cattle nitrate/nitrite poisoning may be suspected based on clinical signs including brownish discoloration of the blood and mucous membranes and a history of exposure to possible nitrate-accumulating forages [2, 4]. However, the characteristic chocolate-colored mucous membranes and blood are not always present and can become less obvious as autolysis proceeds [9].

In cases of suspect nitrate toxicosis-induced abortion, a full necropsy should be performed on the fetus to rule out other potential causes. Ocular fluid from the fetus or cow can be collected for postmortem nitrate/nitrite analysis by ion chromatography with a conductivity detector. This test can be quite reliable when fluid is collected within hours of death and refrigerated. Standard nitrate dipsticks are also available and can be used to make a presumptive diagnosis of nitrate poisoning. Ideally, nitrate levels should approach 30 ppm or greater to be highly suggestive of nitrate/nitrite-associated abortion. However, a compatible history including maternal grazing on potential nitrate-accumulating forages, or some other form of excessive maternal ingestion of nitrates, is also needed [6]. Forage nitrate analysis should also be conducted for confirmation. Forages containing greater than 0.6–1.0% nitrates (6000–10 000 ppm on a dry-matter basis) have been associated with reproductive losses (abortion, stillbirths, and weak calves) [2].

Management and Treatment

Treatment of individual animals with intravenous methylene blue in a 1 or 2% aqueous solution at a rate of 1–2 mg/kg body weight with up to 10 mg/kg in severe cases can be performed. However, due to withdrawal times, cattle should not be sold for slaughter for up to 180 days [9]. Although methylene blue treatment can be effective in individual animals, it is likely cost prohibitive in herd outbreaks. When nitrate/nitrite poisoning is suspected cattle should be removed from pasture. *Propionibacterium acidipropionici* strain P5 (a bacterial feed additive) may be supplemented if exposure to high nitrate concentrations in feed or water is unavoidable. Water should be tested when nitrate contamination is suspected from manure or fertilizer runoff. Water nitrate concentrations should be below 440 mg/l, although acute toxicity is typically not appreciated until water nitrate levels exceed 1300 mg/l. [6]

Ponderosa Pine

Ponderosa pine or western yellow (*Pinus ponderosa*) are common large coniferous trees of the pine tree family (Pinacea) found in all US states west of the Great Plains and throughout western Canada. *P. ponderosa* is characterized by 18- to 28-cm-long, yellow-green needles with two to three needles per fascicle [4, 10]. Ponderosa pine contains a number of compounds that have been associated with toxicity including phytotoxins, mycotoxins, resins, and ligands [4]. Overt toxicosis and death in cattle have occasionally been reported; however, the primary toxicological effects of ponderosa pine needle ingestion in cattle are abortion, retained fetal membranes, and metritis. Open cows, steers, and bulls appear to be unaffected by pine needle

ingestion [11]. Isocupressic acid, a labdane resin acid, is the principal abortifacient compound found in pine needles and the bark of *P. ponderosa* [12]. Two related derivatives of isocupressic acid, acetylisocupressic acid and succinylisocupressic acid, have also been found to be abortifacient following hydrolytic conversion to isocupressic acid in the rumen [13]. Other potentially abortifacient acids present in lesser amounts in ponderosa pine needles include agathic acid, imbricatolic acid, and dihydroagathic acid [14].

Cattle most often ingest early green ponderosa pine needles as well as dry pine needles from the ground in the late fall, winter, and early spring when normal forages are scarce or when they are stressed [4, 10, 11]. Ponderosa pine needle toxicity can result in late-term abortion in cows when ingested primarily during the last trimester. However, abortions have been reported as early as three months of gestation. Cattle may undergo premature parturition anywhere from 2 to 21 days following ingestion of pine needles, although occasionally immediate abortions have been reported [4, 11]. Premature parturition has been induced in cattle fed pine needles 2.2–2.7 kg/day for a period of at least three days [4, 15]. Calves may be born stillborn or premature (small and weak) depending on the stage of the gestation at the time of ingestion [16]. Affected pregnant cows develop edematous swelling of the vulva and mucoid vaginal discharge followed by premature parturition or abortion [4]. Abortions are typically characterized by weak uterine contractions, uterine bleeding, incomplete cervical dilation, dystocia, birth of weak calves, agalactia, and retained placentas. The mechanism of action of isocupressic acid on pregnancy is not completely understood [16]. However, decreased blood flow to the uterus and increased caruncular arterial tone resulting in chronic vasoconstriction of the vascular bed with subsequent fetal parturition are reported. Histologic lesions of placental tissues may include necrosis and hemorrhage secondary to vasoconstriction [11, 16].

Management and Treatment

There are no methods to completely prevent pine needle-induced abortions, although the potential impacts may be minimized. Ideally, pregnant cows should be denied access to pine needles when they are most susceptible to the abortifacient effects of isocupressic acid (third trimester). Pregnant cows should be maintained in adequate body condition when grazing ranges with ponderosa pines to reduce consumption. Adequate food and shelter should be provided for cattle to minimize stress and consumption of pine needles. Calving schedules may be changed to late spring or fall when winter weather conditions are less likely to increase pine needle ingestion [10].

There is no effective treatment for pine needle toxicosis. Supportive care including antibiotic treatment and uterine infusion in cases of placental retention is recommended for cows that abort. Supplemental colostrum and milk may be required for live premature calves [11].

Abortions similar to those caused by ponderosa pine in cattle have been associated with accidental ingestion of Monterey cypress (*Cupressus macrocarpa*) in New Zealand. Monterey cypress is a native of California that has been widely planted throughout Europe and New Zealand. Analysis of *C. macrocarpa* in New Zealand detected elevated concentrations of isocupressic acid. Isocupressic acid is also the primary diterpene acid in *Pinus contorta* (lodgepole pine), *Juniperus communis* (common juniper), Korean pine (*Pinus koraiensis*), and California juniper (*Juniperus californica*), all of which have been associated with late-term abortions in cattle. Cattle abortions have also been attributed to the ingestion of bark from the Utah juniper (*Juniperus osteosperma*) and western juniper (*Juniperus occidentalis*), both of which contain increased concentrations of agathic acid but minimal isocupressic acid [13].

Ateleia glazioviana, *Tetrapterys acutifolia*, and *Tetrapterys multiglandulosa*

Abortions and neonatal mortality in cattle have been attributed to *Ateleia glazioviana*, *Tetrapterys acutifolia*, and *Tetrapterys multiglandulosa* poisoning in Brazil. *A. glazioviana* is a deciduous tree found in western Santa Catarina and northwestern Rio Grande do Sul. Cattle may browse the leaves of this tree when other forages are scarce due to overgrazing or drought. Ingestion of leaves can cause abortions at any time during pregnancy but most occur when green leaves are ingested during the fall and winter months.

T. acutifolia and *T. multiglandulosa* are toxic shrubs that can cause significant outbreaks of abortion and deliveries of weak calves. *A. glazioviana*, *T. acutifolia*, and *T. multiglandulosa* can cause similar clinical signs and lesions. Although abortions can occur at any stage of pregnancy, reports are most often described between six and nine months of gestation. Pregnant cows become lethargic one to three days prior to abortion and occasionally exhibit blindness. Fetal lesions are similar to those observed in adult intoxications. Aborted fetuses may demonstrate subcutaneous edema of the limbs, yellow clear thoracic and abdominal effusion, and myocardial pallor with increased thickness of the right ventricular wall and interventricular septum. The toxin or toxins responsible for abortions and clinical disease in these plants have not yet been identified [17].

Enterolobium spp. and *Stryphnodendron* spp.

Stryphnodendron spp. and *Enterolobium* spp. are trees in the family Fabaceae that produce pods that when ingested have been linked to gastrointestinal signs, photosensitization, and abortions in cattle and other ruminant species in Brazil [18]. *Stryphnodendron obovatum* trees grow in the central-western region of Brazil and are considered to be one of the most important causes of cattle abortions in this region. Cattle readily ingest the fallen pods during the dry season. Abortions may occur during all stages of gestation but are often observed during late pregnancy. The abortive effects of *S. obovatum* were experimentally reproduced by feeding mature pods to seven pregnant cows that were between three and seven months of gestation. Three of seven cows aborted between 20 and 30 days following the start of the study. A fourth expelled a mummified fetus seven months following the experiment. The underlying toxic properties of *S. obovatum* pods were not identified [19]. The abortive properties of *Enterolobium contortisiliquum* have been demonstrated experimentally in guinea pigs but not in cattle [18]. *E. contortisiliquum* and *Dimorphandra mollis* (also of the Fabaceae family) are commonly blamed for abortions in Brazil although this has not yet been proven experimentally [19].

Hairy Vetch (*Vicia villosa*)

Hairy vetch (*Vicia villosa* Roth) (Figure 69.1), originally introduced from Europe as a forage legume and cover crop, can be found throughout much of the USA and in many other countries including Argentina, Australia, and South Africa [4, 20, 21]. Hairy vetch poisoning in cattle is most often associated with the development of multiorgan, granulomatous, and eosinophilic perivascular inflammation most notably of the kidney, heart, adrenal gland, and skin. Clinical findings in affected cattle most often include pruritic dermatitis, conjunctivitis, weight loss, hematuria, and diarrhea. However, reports of sporadic abortion, neurologic dysfunction, subcutaneous swelling of the head, neck, and body, multifocal ulceration of the oral mucous membranes, purulent nasal discharge, rales, cough, and pulmonary congestion have also been reported [21].



Figure 69.1 Hairy vetch (*Vicia villosa*). USDA-NRCS PLANTS Database.

Diagnosis, Mechanism of Action, and Clinical Signs

Hairy vetch poisoning in cattle is most often a diagnosis of exclusion based on clinical signs, histologic findings, and grazing history [20]. Clinical manifestations of hairy vetch toxicity typically develop weeks following ingestion of the plant. The toxic principle of hairy vetch has not been definitively determined, but the pattern and composition of the inflammatory response is highly suggestive of a type IV hypersensitivity reaction. It has been postulated that the inflammatory reaction may be caused by one or more ingested plant constituents (absorbed as haptens or complete antigens) sensitizing antigen-specific lymphocytes that then become distributed throughout the body. Subsequent exposure to these antigens presumably evokes a type IV hypersensitivity response. Another proposed mechanism of toxicity is that vetch lectins bind directly to T-lymphocytes inducing T-lymphocyte activation, lymphokine production, cytotoxicity, and the classic granulomatous inflammatory response [21]. Once clinical signs develop recovery is not promising as there is no effective treatment. Cattle do not appear to be uniformly susceptible to hairy vetch poisoning. Cattle under the age of three years appear to be less prone to clinical disease. Vetch toxicosis has been reported in multiple breeds of cattle, although Holstein and Angus breeds appear to be more commonly affected [4].

Broom Snakeweed

Broom snakeweed (*Gutierrezia sarothrae*) (Figure 69.2) is an invasive, herbaceous, short-lived, woody perennial shrub native to the western rangelands of the USA. The plant can be found from Saskatchewan and Alberta, Canada, south through western Nebraska and Kansas into Texas and central Mexico, and extending to Washington,



Figure 69.2 Broom snakeweed (*Gutierrezia sarothrae*). *Source:* Photograph courtesy of Clarence A. Rechenthin, USDA-NRCS PLANTS Database.

Oregon, California, and Baja California. Heavy growth of broom snakeweed in rangelands is often indicative of poor range management (such as overgrazing) or may be seen in times of drought or following fires [4, 22]. There are two major species of broom snakeweed, *G. sarothrae* (turpentine weed, matchweed, perennial snakeweed) and *Gutierrezia microcephala* (threadleaf broomweed). *G. sarothrae* grows up to 70 cm tall and has a woody base and branching stems with alternate linear leaves and clusters of small yellow flowers. *G. microcephala* is similar in appearance to *G. sarothrae* but has smaller flowers [4].

Broom snakeweeds are toxic and abortifacient to cattle and other livestock species (sheep and goats) [11]. Potentially toxic and abortifacient compounds in broom snakeweed include saponins, furanoditerpene acids, terpenoids, steroids, and flavones [22]. Furanoditerpene acids and flavones found in the leaves of broom snakeweed are similar to isocupressic acid, the abortifacient compound in ponderosa pine. Saponin extracts from threadleaf snakeweed induced abortion in cattle at low concentrations and death in cattle at high concentrations [11]. Abortions caused by broom snakeweed occur mainly when cattle graze during the last trimester of pregnancy [23].

Reported clinical signs include anorexia, weight loss, poor hair coat, listlessness, mucopurulent nasal discharge, abortion, diarrhea, constipation, jaundice, rumen stasis, and death. Abortions can occur throughout gestation and are often accompanied by retained fetal membranes that may lead to uterine infection. Signs of abortion include weak uterine contractions, occasional incomplete cervical dilation, and excessive discharge of vaginal mucus. Low nutrition exacerbates fertility problems caused by broom snakeweed. Supplemental nutrition may enhance degradation and elimination of terpenes. Treatment of clinically affected animals is symptomatic and may include antibiotic use in cows with retained placentas to prevent infection [4, 11].

Snakeweed is typically not very palatable to cattle and is usually only grazed when there is significant grazing pressure due to depleted vegetation. The overall palatability of snakeweed is dependent on the concentrations of plant resins (including terpenes) which accumulate over the growing season. Higher resin concentrations decrease palatability [22]. Proper range management, including providing adequate nutrition for cattle by maintaining the range in good condition, decreases the likelihood of snakeweed toxicosis in cattle. In range regions where snakeweed dominates, management strategies to control the shrub may include the use of herbicides such as Tordon® 22 K (active ingredient: picloram) and Escort® XP (active ingredient: metsulfuron methyl) or prescribed burning. Low nutrition exacerbates fertility problems caused by broom snakeweed, whereas supplemental protein and energy may enhance degradation and elimination of terpenes [11].

Sumpweed (*Iva angustifolia*)

Marshelder, also known as narrowleaf sumpweed, is a native warm-season annual found throughout the south central USA and is a member of the sunflower family (Asteraceae). Reportedly, cattle consuming large amounts of the plant may display premature mammary development, premature milk let-down, and abortion. Sumpweed is seldom eaten by cattle; however, ingestion of young plants during the two- to eight-leaf stage of development has been associated with cattle abortions during four to eight months of gestation and premature calves. The toxic agent/compound is not known [24].

Moldy Sweet Clovers

White sweet clover (*Melilotus alba*) and yellow sweet clover (*Melilotus officinalis*) are biennial legumes (family Fabaceae) commonly grown as forages in the northwestern USA and western Canada. Both species have compound leaflets with serrated edges and pea-like flowers produced on axillary racemes [4]. Ingestion of hay or silage containing spoiled sweet clover (yellow or white) can induce toxicity. Sweet clover naturally contains coumarin which may be converted to the toxin dicoumarol, a potent vitamin K antagonist, by dimerization and oxidation through the actions of fungi such as *Aspergillus*, *Penicillium*, and *Mucor* [25, 26]. Dicoumarol interferes with synthesis of vitamin K-dependent coagulation factors II, VII, IX, and X. These factors are necessary for prothrombin conversion to

thrombin, which polymerizes fibrinogen to fibrin. Impaired fibrin production causes poor stabilization of platelet plugs, predisposing affected animals to hemorrhage [26].

Clinical signs of moldy sweet clover toxicity are often non-specific and vary from excessive hemorrhage following trauma to spontaneous hemorrhages including epistaxis, melena, hemarthrosis, hematuria, and anemia. Hemorrhages typically occur several days to weeks following ingestion of moldy sweet clover. Dicoumarol levels of 20–30 mg/kg in hay are usually required before clinical disease develops [27]. Dicoumarol can cross the placenta causing fetal hemorrhage and has been associated with sporadic abortions in cattle [4, 25, 28].

Clinical signs of dicoumarol toxicity secondary to moldy sweet clover ingestion are indistinguishable from anticoagulant rodenticide toxicosis. A diagnosis of moldy sweet clover poisoning should be suspected when cattle have a history of prolonged consumption of sweet clover combined with compatible clinical signs and prolonged blood clotting times. Dicoumarol analysis of blood from affected animals (live or dead) and analysis of silage or hay can confirm the diagnosis. In cases of clinical disease, affected silage or hay should be removed. Animals demonstrating clinical signs should receive an intramuscular or subcutaneous injection of vitamin K_1 (phytonadione) at a dose of 1.1–3.3 mg/kg body weight every 12 hours for 5 days or until prothrombin time and activated partial thromboplastin time return to normal [26]. In severe cases cattle may be treated with whole blood transfusions (2–4 l of blood per 450 kg) from a donor animal not being fed moldy clover [28]. Blood transfusions are typically indicated when packed cell volumes fall below 12% [26]. Other plants containing coumarin include sweet vernal grass (*Anthoxanthum odoratum*), *Lespedeza stipulacea*, many species of Umbelliferae, and other species of *Melilotus* (melilot) [4, 26]. Herd outbreaks of dicoumarol toxicity have also been reported in cattle ingesting moldy *A. odoratum* hay or silage [26].

Teratogenic Plants

Teratogens are compounds that are capable of inducing developmental malformations or birth defects via insult to a developing fetus. The vast majority of scientific research and investigation in the field of veterinary and human teratology has involved laboratory animals due to their short gestation periods compared with livestock and due to the significant differences in cost involved with project design and the care and maintenance of large-animal subjects [29]. Many factors are involved in the development of teratogen-induced congenital defects.

Teratogen susceptibility can vary greatly among genotypes within the same species which may result in variations in incidence and severity of malformations within the same herd. In order for a teratogenic compound to exert its effect on a fetus, it and/or its metabolites must first reach the conceptus by crossing the placenta. Teratogens, like many other toxins, are dose dependent. Sufficient amounts of the toxic compounds must be ingested by the dam, absorbed into the maternal circulation, and eventually pass through the placenta to the embryo or fetus. Teratogenic compounds in sufficient quantity may result in early fetal loss (abortion or resorption) rather than congenital malformation. In order for a teratogen to cause a specific defect, it must first exert its effect at the appropriate period of time during gestation. For example, for cyclopia to develop in a fetus exposed to *Veratrum californicum*, the toxic plant must be ingested on day 14 of gestation by sheep during the stage of primitive neural plate development [29].

Astragalus and *Oxytropis* (Locoweeds)

Locoweeds, or milk vetches (Figure 69.3), include several species of plants in the genera *Astragalus* and *Oxytropis* [23]. *Astragalus* and *Oxytropis* are members of the Fabaceae (Leguminosae) or pea family. These plants are characterized by their butterfly-like flowers displaying a single large banner petal, two side petals, and two lower fused petals forming a keel. *Astragalus* and *Oxytropis* species have a worldwide distribution. Collectively, locoweeds are considered to be the most detrimental poisonous plant of cattle in the western USA and are responsible for significant economic losses [4, 30].



Figure 69.3 Milk vetch (*Astragalus* spp.). USDA-NRCS PLANTS Database.

Clinical signs of locoweed poisoning are caused by the toxic effects of the indolizidine alkaloid swainsonine found in multiple species of *Astragalus* and *Oxytropis*. Swainsonine is produced by the fungal endophyte *Undifilum oxytropis* (formerly *Embellisia oxytropis*) which can be found in the parenchymal layers of the infected plant seeds [11]. Locoweed poisoning is the chronic condition that develops in ruminants after weeks of ingesting swainsonine-containing plants. Common clinical signs of locoism include depression, incoordination, tremors, weight loss, emaciation, ataxia (staggering), altered mentation, belligerence, aggression, proprioceptive deficits, and eventually death. Reproductive failure, embryo loss, abortion, and occasional birth defects can be associated with locoweed poisoning and are often seen prior to more severe clinical signs in the cow [31].

Mechanism of Action

Swainsonine inhibits the lysosomal enzyme α-mannosidase, which is required for normal hydrolysis of mannose-rich oligosaccharides. In the absence of hydrolysis incompletely processed oligosaccharides accumulate within the cells of multiple organs and tissues resulting in a loss of cellular function and cell death [30, 31]. α-Mannosidosis is the most important acquired lysosomal storage disease in grazing livestock [32]. Microscopic lesions of locoism can be found in many organs and tissues throughout the body; however, cellular vacuolation due to accumulated oligosaccharides is reportedly most pronounced within the brain, liver, kidney, pancreas, and thyroid gland [31].

Teratogenic Effects

Sporadic outbreaks of abortion and congenital limb deformity have been reported in a number of pregnant domestic animal species including cattle, sheep, goats, and horses grazing on species of *Astragalus* and *Oxytropis*. Fetal malformations may include brachygnathia, contracture or overextension of joints, limb rotations, osteoporosis, and bone fragility. The toxic agent responsible for the fetal malformations has not been determined but is likely unrelated to those responsible for the development of lysosomal storage disease [33].

Management and Treatment

Because of the transient nature of locoism, adult animals may recover if they are promptly removed from the swainsonine-containing plants early in the course of their intoxication. Swainsonine is readily cleared from the body, making recovery rapid unless significant tissue damage is present. Although many locoweeds are not highly palatable to cattle, once forced to graze it (usually due to extreme grazing conditions) cattle may develop a preference for the plant that can lead to intoxication [11].

Other Swainsonine-Containing Plants

A number of other swainsonine-containing plant species cause livestock poisonings similar to locoweed poisoning. Some of these include *Ipomea* spp. in Brazil and Africa, *Swainsona* spp. in Australia (swainsonine was first identified in *Swainsona canescens*), and toxic species of *Ipomea*, *Sida*, *Solanum*, *Physalis*, and *Convolvulus* [11, 31]. Swainsonine-containing plants in Brazil include *Sida carpinifolia*, *Ipomea carnea* subsp. *fistulosa*, and *Turbinata cordata*. Fetal malformations and abortions with these plants are similar to those seen with swainsonine-containing locoweed [17, 32].

Other Toxic Effects of *Astragalus* spp.

Over 260 US species of *Astragalus* have been identified as nitrogen accumulators. Nitrogen-containing *Astragalus* spp. can cause both an acute form and a chronic form of nitrate poisoning in cattle. In the acute form cattle develop methemoglobinemia and respiratory distress. Chronic intoxication manifests as generalized weakness, incoordination, and other central nervous system (CNS) signs (knuckling of the fetlocks, goose-stepping, heel-clicking paralysis) and death. Methylene blue treatment can reverse methemoglobinemia in affected cattle but does not prevent mortality, indicating that the cause of death is likely attributed to other factors. Over 20 *Astragalus* species have also been identified as selenium accumulators and may induce selenium toxicosis ("alkali disease") with prolonged grazing [11].

Lupines

Lupines (*Lupinus* spp.), commonly known as blue bonnet, belong to the Fabaceae (legume) family (Figure 69.4). These perennial herbaceous plants are indigenous to North America and range throughout much of the USA. Over 100 species of lupines have been identified. Plants grow up to 1 m high. Leaves are alternate and palmately divided with 5–17 leaflets, which can be smooth or hairy depending on the species. Flowers of *Lupinus* spp. are bonnet-shaped and are present on racemes that are located on a single central stalk. Poisonous varieties of lupine responsible for cattle toxicosis in the USA are predominantly found in the Rocky Mountain region and in states located to the west of the



Figure 69.4 Silver lupine (*Lupinus albifrons* Benth). *Source:* Photograph by Pete Veilleux, East Bay Wilds.

Rockies. Lupines are typically not very palatable to cattle and are most often grazed only when other forages are not readily available [4, 11].

Three specific syndromes in livestock have been attributed to lupine toxicosis in the USA: (i) "crooked calf disease," a teratogenic condition caused by pregnant cows grazing lupines; (ii) acute fatal neurologic disease in adult cattle; and (iii) lupinosis. Lupinosis is not an alkaloid-induced toxicity but is instead caused by cattle grazing lupines infected with the fungus *Phomopsis leptostromiformis* that produces the mycotoxin phomopsin. Clinical signs of phomopsin poisoning may include severe hepatic, renal, and muscle disease in cattle and sheep [4]. Clinical signs of the neurologic syndrome in adult cattle include muscle weakness, agitation, frequent urination and defecation, depression, frothing at the mouth, relaxation of the third eyelid, muscle fasciculations, ataxia, lethargy, collapse, and sternal recumbency followed by lateral recumbency, respiratory failure, and death [11].

Maternal ingestion of toxic lupines during days 40–70 of gestation results in significant fetal malformations depending on the concentration of teratogenic compounds present in the plant [29]. Alkaloids responsible for fetal teratogenic effects (crooked calf syndrome) include anagyrine, ammodendrine, and *N*-methyl-ammodendrine [11].

The most commonly observed lupine-induced fetal malformation is arthrogryposis of the forelimbs (Figure 69.5), which may be accompanied by scoliosis, torticollis, lordosis, kyphosis, or palatoschisis (cleft palate) [11, 34]. Muscles from affected limbs are often hypoplastic or atrophied. Muscle atrophy is attributed to disuse of the affected limb(s) in utero [34]. The mechanism of action for teratogenic fetal effects is an alkaloid-induced decrease in fetal movement secondary to desensitization of skeletal muscle-type nicotinic acetylcholine receptors. Decreased or inhibited fetal movement results in skeletal malformations including skeletal muscle contractions and cleft palate formation [11].



Figure 69.5 Arthrogryposis of hindlimbs (crooked calf).

Management and Treatment

Prevention and management of lupine poisoning can be achieved by coordinating grazing periods of susceptible pregnant cows with growth stages of the plant, ensuring that susceptible pregnant cows do not graze during the flower and pod stages of growth when anagyrine concentrations are at their highest. Other effective measures may include changing the calving periods from spring to fall and by instituting intermittent grazing between lupine-free pasture and lupine-infested pasture to break the cycle of lupine ingestion. Broadleaf herbicides such as 2,4-D can also be utilized to reduce lupine numbers in heavily infested pastures, although lupine populations commonly reestablish due to the presence of resident seeds within the soil.

Treatment of clinically ill cattle involves supportive and symptomatic care. Animals displaying signs of intoxication should not be stressed and should be allowed to rest. Because the toxic alkaloids are rapidly excreted in the urine, affected adult animals will often make a full recovery within 24 hours. No treatment is available for severe fetal malformations. However, calves with mild contracture defects, of the forelimbs primarily, may recover if the elbow joint can be locked into place within one week of birth [11].

Poison Hemlock (*Conium maculatum*)

Poison hemlock, also known as European hemlock, spotted hemlock, and California fern, is a biennial perennial member of the parsley/carrot (Apiaceae, formerly Umbelliferae)



Figure 69.6 Poison hemlock (*Conium maculatum*). USDA-NRCS PLANTS Database.

family found all across the USA (Figure 69.6). *Conium maculatum* is one of the most toxic members of the plant kingdom [35]. It was originally introduced to North America from Europe as an ornamental plant and is also native to Asia but can be found throughout North and South America, North Africa, Australia, and New Zealand. Poison hemlock is a tall branched plant with distinctive purple spots along its stems, multiple small white five-petaled flowers, a single taproot, and a distinctive mousy odor [4, 31, 36].

Poison hemlock contains eight known piperidine alkaloids [36]. The two major toxic alkaloids are γ-coniceine found in immature growing plants and coniine found in mature plants and seeds [4, 31, 36]. However, all vegetative organs, flowers, and fruits contain alkaloids [35]. Alkaloid levels vary based on environmental factors (such as temperature and moisture), stage of growth, and geographic location. Fresh drying the plant in the sun for seven days results in significantly decreased levels of the toxic piperidine alkaloids [31].

Poison hemlock is highly toxic to cattle and in acute poisoning cases clinical signs may develop rapidly following ingestion (30 minutes to 1 hour). Reported clinical manifestations include depression, abdominal pain, mydriasis (dilated pupils), frequent urination and defecation, increased salivation (ptyalism), lacrimation, muscle tremors and fasciculations, incoordination and ataxia, respiratory distress, recumbency, collapse, and death. Animals surviving acute poisoning usually recover but abortions may result. The teratogenic effects of *C. maculatum* are most often appreciated in cases of chronic ingestion by pregnant cattle. However, fetal malformations have also been described in piglets and lambs [36]. Ingestion of the plant between days 40 and 70 of gestation can result in a wide array of skeletal malformations in the neonate [4]. Reported teratogenic malformations are indistinguishable from those caused by lupines and tobacco species. Fetal malformations may include torticollis (twisted neck), scoliosis (vertebral column curvature), palatoschisis (cleft palate), arthrogryposis (multiple joint contractures), and excessive flexure of the carpal joints (crooked calf disease) [35, 36].

Mechanism of Toxicity in Acute Poisoning

Conium alkaloids, specifically γ-coniceine, coniine, and *N*-methylconiine, act as non-depolarizing blockers (akin to curare) resulting in paralysis of motor nerves through their action on the medulla. It has been proposed that these alkaloids initially stimulate the skeletal muscles and produce subsequent neuromuscular blockade through their action on nicotinic receptors [4, 35, 36]. Death occurs when the phrenic nerve is affected and respiratory muscles become paralyzed [36].

Mechanism of Teratogenicity

Poison hemlock-related fetal malformations, such as joint contractures, are believed to result from fetal immobility induced by neuromuscular blockade of nerves. Palatoschisis in calves may be secondary to the loss of fetal mandibular and tongue movements, which are both required for palatal shelves to become closer in the developing fetus. In the absence of jaw and tongue movements, the tongue permanently occupies the site where palatal shelves should fuse [36, 37].

Management and Treatment

Treatment of poison hemlock toxicosis relies largely on supportive care. In cases of acute toxicity, gastric lavage and activated charcoal treatment may be performed on individual animals. Control of the weed can be managed through the use of broadleaf herbicides, although populations will reestablish via seed reserves in the soil [11].

Tobacco Poisoning

Nicotiana glauca (tree tobacco), *Nicotiana tabacum* (cultivated tobacco), *Nicotiana trigonophylla* (wild or desert tobacco), and *Nicotiana attenuata* (wild or coyote tobacco) are members of the nightshade (Solanaceae) family. Wild tobacco varieties are indigenous to the southwestern USA while cultivated tobacco grown in the USA is predominantly raised in the southeastern states. Tobacco is an

herbaceous branching annual that grows between 0.3 and 1.2 m in height, with hairy stems and leaves and fragrant tubular flowers with five parts. Tree tobacco (*N. glauca*) is an evergreen shrub or small tree growing 2–6 m tall with alternate bluish-green hairless leaves. Flowers are 5 cm long, tubular, and produced on the leafless branches [4].

Tobacco contains numerous alkaloids (over 40), most of which are neurotoxic pyridine alkaloids including nicotine. Animals may become intoxicated by grazing the plant or ingesting cut stalks [38]. In adult cattle clinical signs of acute toxicity may include excitation, followed by depression, respiratory failure, and death in severe cases. Treatment of affected animals is symptomatic. The principal toxins responsible for tobacco toxicosis in cattle are nicotine and anabasine. *N. glauca* produces the piperidine alkaloid anabasine, a potent nicotinic acetylcholine receptor agonist. Anabasine is acutely toxic as well as teratogenic [38]. Ruminants are relatively more tolerant of the neurotoxic alkaloid effects of nicotine. However, the teratogenic anabasine can induce fetal skeletal malformations including arthrogryposis, palatoschisis, torticollis, lordosis, and kyphosis. Fetal defects resulting from tobacco intoxication occur when cattle ingest the plant between days 30 and 60 of gestation.

The fetal malformations caused by tobacco toxicosis are indistinguishable from those induced by poison hemlock, locoweeds, and lupine poisoning. This similarity of teratogenic fetal malformations is attributed to the analogous molecular structures of anabasine and coniine (principal toxin of *C. maculatum* and lupines) and its analogs [4].

At low doses anabasine stimulates depolarization of postsynaptic membranes of ganglia and at high does causes neuromuscular blockade. Like poison hemlock and lupine toxicosis, the mechanism of teratogenicity is believed to be caused by the neuromuscular blockade of nerves and subsequent decrease in intrauterine fetal movement resulting in the development of joint contractures, palatoschisis formation, and other skeletal malformations [39, 40]. In acute toxicosis, cattle should be removed from contaminated fields or pastures. In severe cases, supportive and symptomatic treatment is recommended for affected animals and may include gastric lavage [28].

Veratrum californicum (False Hellebore)

V. californicum grows in dense, sharply defined stands in high moist meadows or hills [29]. Individual plants grow approximately 2–3 m tall and have short rootstalks and alternate smooth, broad, parallel-veined oval leaves that are present in three ranks. Numerous toxic alkaloids have been identified in *V. californicum*; however, the jervine alkaloids including cyclopamine, jervine, and cycloposine are associated with the plant's teratogenic effects [4, 39]. Among the toxic alkaloids, cyclopamine is present at the highest levels and is the principal teratogenic alkaloid [29].

Teratogenic effects are predominantly seen in sheep but fetal malformations have also been reported in goats and cattle [33]. The teratogenic alkaloids exert their effects by interfering with the sonic hedgehog (SHH) signal transduction pathway, by inhibition of neuroepithelial cell mitosis and migration, and by decreasing chondrocyte proliferation. In pregnant ewes, ingestion of *V. californicum* on days 12–14 of gestation (most notably day 14) results in cyclopia and prolonged gestation. Embryo death can result from maternal exposure on days 19–21. Cleft palate results from maternal exposure on gestational days 24–30 and metacarpal and metatarsal defects occur when ewes are exposed on days 28–31 of gestation [39].

Ingestion of *V. californicum* in pregnant cows between days 12 and 30 of gestation may cause fetal malformations, including cleft palate, harelip, brachygnathia, hypermobility of the hocks, syndactyly, and decreased numbers of coccygeal vertebrae. When pregnant cows ingest the plant in later stages of pregnancy (days 30–36), teratogenic alkaloids inhibit growth in length of metacarpal and metatarsal bones [33].

There is no specific treatment for *V. californicum*-induced fetal malformations. However, malformations can be prevented by removing pregnant animals from *V. californicum*-infested pastures and ranges during the first trimester of pregnancy [4].

Mimosa spp.

Mimosa tenuiflora and *Mimosa ophthalmocentra* are perennial trees or small shrubs native to Brazil. *M. tenuiflora* and possibly *M. ophthalmocentra* (Fabaceae, Mimosoideae) can induce fetal malformations and embryonic death in goats, sheep, and less frequently cattle. Affected calves, lambs, and kids may be born with an array of malformations, which can include arthrogryposis, micrognathia, cheiloschisis (harelip), palatoschisis, kyphosis, lordosis, torticollis, blindness, microphthalmia, corneal opacity, ocular dermoids, acephaly, bicephaly, hydranencephaly, glossal hypoplasia, meningocele, and syringocele. The toxic principle of *M. tenuiflora* is unknown, but tryptamine-derived alkaloids have been isolated from the leaves and seeds [17].

Mycotoxins

Mycotoxins are naturally occurring compounds or secondary metabolites produced by fungi that cause deleterious effects (mycotoxicoses) when ingested or when susceptible animal species are exposed to them. Mycotoxisosis in livestock occurs when animals graze on infected grasses or crops or are fed infected grains. Chemical analysis via thin-layer chromatography, gas and high performance liquid chromatography, and mass spectrometry can accurately detect the concentrations of various mycotoxins in properly collected and prepared samples [41, 42].

Ergot Alkaloids

Ergot alkaloids include more than 80 indole compounds and are associated with two mycotoxicoses in livestock species: fescue toxicosis and ergotism [2]. Both conditions may share similar clinical manifestations and in many cases may be indistinguishable from one another [43]. Ergot alkaloids are produced by the mold *Claviceps purpurea* on wheat, rye, and other cereal grains and by the endophyte *Neotyphodium coenophialum* growing on tall fescue (*Lolium arundinaceum* [Shreb]). Ergot alkaloids have similar molecular structures to the biogenic amines norepinephrine, serotonin, and dopamine and act as agonists at various serotonin receptors [44, 45]. This agonistic effect is thought to be responsible for clinical manifestations of ergot alkaloid toxicosis. In both fescue toxicosis and ergotism the principal ergot alkaloid believed to be most responsible for clinical disease is ergovaline. Ergovaline is a potent α_2-adrenergic agonist on arterioles and other blood vessels causing vasoconstriction [42].

Tall Fescue

Fescue toxicosis is a costly grass-related intoxication of livestock in the USA and throughout many other countries. Fescue toxicosis is a descriptive term used for several clinical syndromes (summer slump, fescue foot, and fat necrosis) attributed to ingestion of endophyte-infected tall fescue [42].

Tall fescue (*L. arundinaceum* [Shreb]) is a cool-season perennial grass grown in temperate climates and is one of the most abundant forage crops in the continental USA [46]. In cases of fescue toxicosis, livestock ingest the fungal endophyte *N. coenophialum* (formerly *Acremonium coenophialum* and *Epichloe typhina*) that grows within the intercellular spaces of the leaf sheaths, stems, and seeds of tall fescue grass [2]. Tall fescue and its endophyte *N. coenophialum* share a mutually beneficial relationship. The endophyte produces toxins (ergot alkaloids, loline alkaloids, and peramine) that are distributed throughout the plant, making it more resistant to drought, parasitism, and fungal infection [42]. The endophyte of tall fescue produces an array of ergot alkaloids but ergovaline is the primary ergopeptine alkaloid believed to be responsible for inducing toxicosis. Ergot alkaloids are highly concentrated within the seeds of tall fescue and levels are greatest in the summer and early fall when seed heads are present [46]. Drought and rainy conditions as well as fertilization with nitrogen- and phosphorus-based fertilizers tend to increase ergovaline concentrations [42].

Several syndromes including summer slump, fescue foot (Figure 69.7), and fat necrosis (lipomatosis) are associated with fescue toxicosis in cattle. The most costly of the three is summer slump. As the name implies, clinical manifestations of this condition are most often appreciated in summer months when the ambient temperature is above 31 °C [42]. Affected cattle typically have a rough hair coat, unthrifty appearance, reduced feed intake, decreased milk production with lower weaning weights of calves, reduced conception rates, agitation/nervousness, ptyalism, and increased respiratory rates [42, 47].

Mechanism of Action

Ergot alkaloids are dopamine D_2 receptor agonists and mimic the endogenous tonic inhibition of pituitary lactotropes by dopamine, thereby inhibiting prolactin secretion from the anterior pituitary gland [42]. Decreased serum prolactin is a relatively consistent finding in livestock feeding studies involving ergot alkaloid-infected forage. Therefore serum prolactin concentrations may be utilized as a diagnostic indicator of endophyte toxicosis [48].



Figure 69.7 Fescue foot: dry gangrenous dermatitis secondary to "fescue toxicosis." *Source:* Photograph courtesy of Dr. Jim Cooley.

Reproductive Effects

The hormone prolactin plays an important role in mammary gland growth, milk production, corpus luteal function, gonadotropin secretion, lipogenesis, copper homeostasis, and immune modulation. Decreased prolactin production caused by the D_2 dopaminergic effects of ergovaline and other ergot alkaloids can cause agalactia at parturition, most notably in horses. Cattle and other ruminants produce a placental lactogen that can overcome the initial lack of prolactin stimulation at the time of parturition. However, lower concentrations of prolactin coupled with decreased feed intake can cause decreased milk production in these species [42]. Prolactin also plays an important role in gonadotropin secretion and corpus luteal function and development. Decreased concentrations of luteinizing hormone and prostaglandins have been documented in animals exposed to ergot alkaloids [48]. Ingestion of endophyte-positive fescue and ergopeptide exposure has been associated with low progesterone production in cattle and horses. Decreased progesterone, as well as other imbalances in reproductive hormones, can result in difficulty maintaining early pregnancy in cattle and in late pregnancy problems in mares [42].

In addition to their dopaminergic effects, ergot alkaloids act as potent serotonergic agonists and α_2-adrenergic agonists on blood vessels, particularly arterioles. Peripheral vasoconstriction of vessels is believed to contribute to the hyperthermia exhibited in some cases of summer slump in the warm summer months along with dysregulation of the hypothalamic thermoregulatory center due to decreased prolactin secretion. In winter months, toxicosis may result in fescue foot, a condition caused by peripheral vasoconstriction of vessels of the distal limbs secondary to the combined effects of ergovaline and cold environmental temperatures. The tail switch and ear tips as well as the distal limbs (most often the hindlimbs between the coronary band and fetlock) may also be affected [42].

Management and Treatment

Cattle experiencing clinical signs of summer slump should be removed from endophyte-contaminated pasture or their diet should be diluted by feeding supplemental grain or nonfescue forages [42]. Cattle with gangrenous dermatitis (fescue foot) can be treated with antibiotics to prevent secondary infections. Shelter or wind breaks may be beneficial in minimizing the effects of cold weather. Domperidone, a D_2 dopamine antagonist, is used to treat fescue toxicosis in mares with agalactia and prolonged gestation. Experimentally, domperidone has been shown to ameliorate some clinical signs of fescue toxicosis in heifers, including reversal of decreased weight gain and increased circulating concentrations of progesterone. These results suggest that treatment of cattle with fescue toxicosis may have clinical and economic merit [49].

Ergotism

Ergot is a general term referring to a group of parasitic fungi belonging to the genus *Claviceps*, most notably *C. purpurea*, that infect rye, barley, wheat, oats, and other cereal grains [44]. Infection of grains with *C. purpurea* results in colonization of the grain ovary wall or base with fungal mycelia. The mycelia produce a sticky exudate called honeydew containing conidia (asexually produced spores). Following germination within the hardened honeydew (sclerotia), conidia can be transferred to and infect other seeds by insect vectors or direct contact [44, 45]. Signs of ergotism – the various toxic effect(s) of ergot alkaloids produced by *Claviceps* spp. – develop secondary to the ingestion of toxic levels of ergot-infected grains [44].

Clinical signs of ergotism are primarily caused by the ergopeptine class of ergot alkaloids, which include ergotamine, ergocristine, ergosine, ergocornine, ergocryptine, and ergovaline. Ergot alkaloids act as serotonin receptor agonists due to the similarities of their molecular structures with the biogenic amines dopamine, serotonin, and histamine [44, 45]. Vasoconstriction results from the agonistic activity of ergot alkaloids. Other potential effects include uterine stimulation and dopaminergic activity at D_2 receptors resulting in decreased prolactin secretion [45]. Documented cases of ergotism in the USA are uncommon because milling and cleaning processes are successful in removing ergot-infected grains [41].

Ergot alkaloid toxicity is known to cause four distinct disease forms in livestock: (i) cutaneous and gangrenous lesions on the tail and extremities, (ii) hyperthermia and production loss, (iii) reproductive failure, and (iv) the less common and frequently debated neurologic form [2, 45]. The gangrenous form of ergot poisoning represents the chronic form of intoxication by ergot-producing fungi. Clinical signs of gangrenous ergotism include acute lameness with swelling and hyperemia. Lesions present most often in the rear limbs, although all four limbs may be affected in some cases; however, necrosis is usually restricted to below the fetlock but occasionally extends to the metatarsals [20]. Vasoconstriction of small arteries and arterioles due to the potent α_2-adrenergic agonistic effects of ergovaline causes ischemia and necrosis of the distal limbs, ear tips, and the distal third of the tail. This gangrenous form is most often associated with winter months due to exacerbation of the vasoconstrictive effects by cold

weather [45]. The hyperthermic form of the disease is the most commonly reported form of ergotism and is seen most often in summer months when the weather is warm [44]. The neurologic form of ergotism appears to be rare and may be associated with acute intoxication with large doses of ergot alkaloids [45].

Management and Treatment

When ergotism is suspected, infected grains should be removed. No effective treatment is available for ergotism in cattle, although animals with the hyperthermic form should recover given time [45].

Aflatoxins

Aflatoxins (B_1, B_2, G_1, and G_2, named for their respective blue or green fluorescence under ultraviolet light) are a group of structurally related difuranocoumarin compounds produced primarily by *Aspergillus flavus* and *Apergillus parasiticus*. Aflatoxins can be found throughout the world and can commonly contaminate animal feed and crops in warm subtropical and tropical climates under appropriate conditions [40, 41, 43]. Grains stored under high moisture (>14%), at warm temperatures (>20 °C), or those improperly dried are at risk of becoming contaminated [50]. *A. flavus* strains invade damaged plant tissue and predominantly produce aflatoxin B_1 which is considered to be the most toxic and carcinogenic aflatoxin [43].

All animals are susceptible to the toxic effects of aflatoxins, although differences in species sensitivities do exist. Susceptibility can vary with breed, age, dose, nutritional status, and length of exposure [50]. Cattle and other ruminants are considered to be relatively resistant in comparison with monogastric species. However, young calves are at greater risk of developing clinical toxicosis compared with adult animals [43].

Aflatoxins are primarily hepatotoxic but can also have immunosuppressive, carcinogenic, mutagenic, and teratogenic effects [43]. Some aflatoxins have been shown to cross the placenta in laboratory animals and humans. Decreased male fertility via decreased spermatozoa production, embryo loss, fetal death, and possible teratogenic effects have been reported with aflatoxicosis, most notably with aflatoxin B_1 and G_1 in animals [51]. Abortions in cattle are uncommonly reported with aflatoxicosis, but sporadic herd outbreaks have been attributed to ingestion of feeds contaminated with aflatoxin B_1, B_2, G_1, and G_2 [43]. However, the negative reproductive impacts do not appear to be due to any direct activity of aflatoxins in the oocyte or embryo but instead are attributed to altered maternal homeostasis [41].

Treatment

There is no specific treatment for aflatoxicosis. Instead treatment of affected cattle involves supportive and symptomatic care. Contaminated feed should be removed and replaced with aflatoxin-free feeds [51].

Zearalenone

Zearalenone is a potent estrogenic mycotoxin produced by the fungus *Fusarium graminearum* and multiple other species of *Fusarium*. Zearalenone contaminates cereal grains including corn and to a lesser extent wheat, barley, sorghum, millet, rice, and oats under specific environmental and storage conditions [39]. Zearalenone can have profound effects on reproductive function due to its estrogenic actions. Clinical signs attributed to zearalenone toxicity are most often reported in swine, although signs may also be observed in ruminant species as well. Clinical signs of zearalenone toxicity mimic those of estrogenic stimulation and can include decreased fertility, abnormal estrous cycles, vulvar swelling, vaginitis, reduced milk production, and mammary gland enlargement. Heifers appear to be more susceptible to the effects of zearalenone than adult cows [41, 52]. However, in pregnant cows the potential for abnormal embryonic or fetal development with possible fetal death, abortion, and dystocia exists [2].

Zearalenone and its derivatives (α- and β-zearalenol) are believed to exert their toxic effects by competitively binding to specific estrogen receptors and by modification of steroid metabolites. Zearalenone and α- and β-zearalenol have chemical structures closely resembling naturally occurring estrogen [53]. Zearalenone and its metabolites can directly bind with cytoplasmic estradiol-17β receptors and translocate receptor sites to the nucleus where stimulation of RNA leads to protein synthesis and clinical signs of estrogenism [52]. β-Zearalenol appears to be the predominant zearalenone metabolite in cattle as opposed to α-zearalenol which predominates in most other species including swine [2].

Treatment

Treatment of zearalenone intoxication relies on removal of contaminated feedstuff and replacement with high-quality clean feeds. Clinical signs will typically resolve within three to seven weeks. Prevention of *Fusarium* spp. growth and subsequent zearalenone production can be achieved by maintaining low moisture concentrations (15–16%) [52].

Phytoestrogens

Phytoestrogens are biologically active non-steroidal plant compounds that are similar in structure or function to mammalian estrogen and can compete for estrogen receptors on cells [4, 54]. Plant estrogens can have broad effects on both human and animal populations. Phytoestrogens are found in a number of legumes including alfalfa (*Medicago sativa*), soybeans (*Glycine max* L.), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*), white clover (*Trifolium repens*), aslike clover (*Trifolium hybridium*), and burr medic (*Medicago* spp.) among others [4, 39, 55].

Concentrations of phytoestrogens in plant material can vary significantly. Estrogenic compounds are often produced in response to environmental or physiologic plant stressors such as fungal infection, insect infestation, and animal predation. Phytoestrogen concentrations have also been shown to increase in cool wet conditions [54].

Over 100 phytoestrogen molecules have been identified. Phytoestrogens are subdivided into broad categories based on their chemical structure. Categories of phytoestrogens include isoflavones, flavones, coumestans, stilbenes, and lignans [54, 56]. Isoflavone compounds can be found in high concentrations in soybean, red clover, and white clover. Coumestrol (potent estrogenic phytoestrogen) can be found in alfalfa as well as white clover and soybean sprouts [54]. Stilbenes are found in cocoa- and grape-containing products and lignans are prevalent in flax seed [56, 57].

Once ingested, phytoestrogens are absorbed in the rumen by resident microbial activity. Because of structural similarity with endogenous hormones, phytoestrogens can bind to nuclear receptors [56]. Although phytoestrogens may mimic estradiol-17β (E2), their effects may not always be similar [58]. The affinity phytoestrogens have with ERα receptors and ERβ receptors is relatively weak in comparison with endogenous E2 [56]. However, phytoestrogens can be present in much higher concentrations in the body (as much as 100-fold higher) than endogenous estrogens, allowing for potentially profound effects. Phytoestrogens may exert agonistic, partial agonistic, or antagonistic effects depending on the concentration of E2 [56, 57].

The toxic effects of phytoestrogens are dependent on dose, route of exposure, duration of exposure, and timing of exposure [57]. Organ systems in young animals appear to be more sensitive to phytoestrogens than adults. Cattle infertility has been reported in herds fed red clover silage containing isoflavones and alfalfa-containing coumestans [58]. However, cattle appear to be less sensitive than sheep to the phytoestrogenic effects of clover forages [54]. The effects of high concentrations of phytoestrogens may include clinical signs of hyperestrogenemia (nymphomania, cystic ovaries, swollen genitalia, infertility, impaired pregnancy maintenance) or antiestrogenic effects such as gonadal hypoplasia and signs of anestrus, depending on the action of the phytoestrogen on estrogenic receptors.

Uterine endometrial release of prostaglandin (PG)$F_{2\alpha}$ is regulated by oxytocin, progesterone, and estradiol (E2) in ruminants and induces luteolysis and corpus luteum regression. In a study conducted by Mlynarczuk et al. [55] the phytoestrogens coumestrol, daidzein, and genistein were shown to stimulate the genes responsible for synthesis of oxytocin precursors (neurophysin-I/oxytocin) and post-translational synthesis of oxytocin, PGA (peptidyl-glycine-α-amidating monooxygenase) in granulosa cells, and luteal cells in cattle. These phytoestrogens stimulated oxytocin secretion from the ovarian follicle and corpus luteum. It is speculated that phytotoxin-induced oxytocin release during early pregnancy or implantation may result in early luteolysis, formation of persistent corpus luteum, and subsequent impaired pregnancy maintenance and early embryonic death [54, 55].

Management and Treatment

In order to prevent or minimize potential reproductive problems, forages can be analyzed for isoflavones and coumestrol concentrations and feeds containing low or no levels can be substituted for high phytoestrogen-containing forages. Once phytoestrogen-affected forages have been eliminated from cattle rations, animals will typically resume normal reproductive cycling within a period of four to six weeks [54].

Trace Minerals and Heavy Metals

Heavy metals and trace minerals are common environmental contaminants and when present in sufficient levels can cause significant health and reproductive impacts in domestic livestock. Heavy metals include elements that under appropriate conditions play a necessary role for normal body function, such as iron, copper, zinc, and chromium, as well as toxic elements that have no biological functions. Many minerals are crucial for animal health, survival, and production due to the essential roles they play in the physiological, structural, and regulatory functions of the body. However, ingestion of excessive amounts of minerals may lead to acute poisoning or chronic poisoning, when animals ingest lower concentrations over prolonged periods of time [59].

Potentially toxic heavy metals include lead, mercury, cadmium, chromium, arsenic, and nickel. Environmental

pollution with trace elements, toxic metals, and metalloids is often attributed to mining, industrial wastes, urbanization, and intensification of farming practices (pesticide and herbicide activity). Transfer of heavy metals from contaminated soils into plants and successive levels of the food chain can be a significant health risk in both animals and humans. Clinical manifestations of trace mineral and heavy metal intoxication can vary depending on the element involved, health of the animal, and degree of exposure. Several heavy metals have been identified as potentially teratogenic and abortifacient in animals [60]. However, in cattle the most commonly reported heavy metal toxicities involve lead and arsenic.

Arsenic

Arsenic (As) is a ubiquitous metalloid element present in multiple forms, the most common oxidative states being the As^{3+} and As^{5+} valence forms [61]. Arsenic can be found in both organic and inorganic forms, although inorganic arsenite is the most toxic form of the element.

Arsenic is predominantly used in the manufacture of pesticides, wood preservatives, glass, alloys, semiconductors, and pharmaceuticals. Environmental contamination of arsenic has been attributed to smelting of lead, copper, and nickel, combustion of fossil fuels, manufacturing of arsenic-containing compounds, and overuse or improper storage of arsenic-containing herbicides and insecticides [62].

Peracute exposure to large doses of inorganic arsenic is often fatal and may result in death within minutes to hours depending on dose. Clinical signs of acute toxicosis may be profound and include colic, bloody diarrhea, neurologic signs such as ataxia and incoordination, weakness, and collapse followed by death. Gross postmortem lesions in cattle may include abomasal hyperemia, increased intraluminal intestinal fluid, edema of the rumen, omasum, reticulum, and abomasum, ruminal mucosal necrosis, and gastrointestinal hemorrhage [61].

Mechanism of Action

Absorption of arsenic compounds depends on the particle size and its aqueous solubility. Soluble forms of arsenic are readily absorbed via both passive and active means [61, 62]. In pregnant animals, inorganic arsenic crosses the placenta and accumulates in the neuroepithelium of the developing fetus. Organic arsenic accumulates in the placenta but does not appear to cross the membrane. Laboratory animal studies have demonstrated the embryotoxic effects of high doses of inorganic arsenic, with development of teratogenic defects such as cleft palate, encephalocele, microphthalmia in hamsters and failure of limb bud development, prosencephalon hypoplasia, and somite abnormalities in rats and mice exposed during early gestation. Arsenic at lower concentrations can also have significant placental and fetal toxic effects [40, 62]. However, reports of abortion in cattle with subclinical or chronic arsenic toxicosis are sporadic.

Diagnosis, Treatment, and Prevention

A presumptive diagnosis of arsenic toxicosis is often based on an appropriate clinical history of exposure and suspicious clinical signs. Necropsy lesions can also be suggestive. However, definitive diagnosis requires demonstration of elevated liver and kidney arsenic concentrations. Arsenic levels of approximately 8–10 ppm in fresh liver and kidney and 2–4 ppm in liver and kidney from autolyzed carcasses (several days old) are diagnostic.

Early recognition and medical intervention is imperative for successful treatment of arsenic intoxication cases. In cattle not showing clinical signs, gastric lavage with a saline purgative and 20–30 g sodium thiosulfate administered orally or intravenously in a 10–20% solution may be effective. Injection of dimercaprol 1.5–5 mg/kg intramuscularly (IM) two to four times daily for 10 consecutive days should also be administered if over four hours have elapsed since time of exposure. Additional treatment with sodium thiosulfate 30–40 mg/kg intravenously (IV) two to four times daily until recovery (typically two to four days) is also recommended [61].

Cadmium

Cadmium (Cd) is a divalent transition metal with chemical properties similar to those of zinc [63]. Unlike many minerals, cadmium has no essential physiologic or biochemical function in the body and is highly toxic to animals and humans [59]. Cadmium compounds are widely utilized in industrial applications. They are used in iron and steel plating and can be found in batteries, semiconductors, solders, plastic stabilizers, and solar cells. Environmental contamination of water and soil is often secondary to industrial pollution. Cadmium may enter the environment from steel and iron production, zinc refining and smelting, mine wastes, coal combustion, improper battery disposal, and the use of sewage sludge and rock phosphate as fertilizers [63]. Batteries are considered to be one of the most common sources of cadmium intoxications in cattle in the USA. However, phosphate fertilizers and sewage sludge are also important sources of cadmium toxicosis in cattle [64].

Some plants readily accumulate cadmium from contaminated soil, making it available for consumption by grazing animals [59, 63].

Cadmium, once ingested, is primarily absorbed through the respiratory system and digestive tract and accumulates mainly within the liver and kidneys. Gastrointestinal absorption of cadmium is much less than other divalent cations (zinc and iron). However, intestinal absorption can be increased in animals with iron deficiency. Following absorption, the metal circulates either in red blood cells or bound to albumin in the plasma. Cadmium interacts with the metabolism of essential minerals such as calcium, zinc, iron, copper, and selenium. Cadmium ions can displace divalent metals such as zinc from their binding sites on metallothionein (major metal-binding protein) [63, 64]. Metallothionein functions in cadmium detoxification through high-affinity binding with the mineral that results in a toxicologically inert compound [59, 64]. Cadmium along with other heavy metals (Cu, Zn, and toxic mercury) induces metallothionein synthesis in multiple organs including the liver and kidneys. However, because metallothionein's action is limited, excessive amounts of cadmium ingested acutely or over time can exceed metallothionein's ability to bind it and significant multiorgan damage can develop [59]. In the liver, cadmium toxicity results in hepatocellular apoptosis and necrosis [63]. High levels of dietary cadmium can cause decreased feed intake, poor weight gain, anemia, decreased bone absorption, and abortions [64]. There is no specific treatment for cadmium poisoning in livestock and therefore exposure should be minimized or prevented in the environment and in feeds [63].

Lead

Lead poisoning in cattle often results from environmental contamination. Cattle may become acutely intoxicated from the ingestion of large quantities of lead-containing materials, licking old storage batteries, licking or ingesting lead-containing paint chips on outbuildings or from discarded paint cans, eating lead-containing lubricants, and drinking water from lead-containing water pipes [6]. Chronic exposure most often occurs from ingesting forages grown on polluted pastures in the vicinity of mines or industrial sites.

Only about 2–10% of ingested lead is absorbed by the digestive tract and distributed throughout the body [6]. Absorbed lead is slowly excreted in bile, milk, and urine and is deposited in tissues, predominantly in the kidney, liver, brain, and bone.

Lead induces toxic effects via multiple mechanisms including combining with calcium- and zinc-binding proteins, random hydrolysis of nucleic acids, and by inducing RNA catalysis via activation of ribosomal 5S RNA [65]. Lead interferes with protein/hemoprotein biosynthesis, inhibits mitochondrial and membrane enzymes, and causes deficits in cholinergic, dopaminergic, and glutamatergic functions [40]. Lead disrupts calcium homeostasis, causing the accumulation of calcium in lead-exposed cells and mitochondrial release of calcium inducing apoptotic cell death.

In adult cattle acute toxicity typically causes death within 12–24 hours. When poisoning is less acute animals may survive four to five days. Clinical signs may include cortical blindness, delayed menace response, delayed withdrawal reflex, decreased glossal tone, ptyalism, frothing at the mouth, champing of the jaws, respiratory difficulty, tachycardia, ataxia, head pressing, convulsions, circling, and recumbency. Signs of subacute poisoning may include depression, anorexia, bruxism, colic, diarrhea, ruminal atony, CNS signs, and abortion [40, 65].

Lead readily crosses the placenta and can pass through the immature blood–brain barrier of developing fetuses where it concentrates in the CNS [6, 40, 65]. Lead can also accumulate in the placenta during times of fetal stress. The toxicity of lead is primarily attributed to its ability to mimic and substitute calcium. Effects of lead on the developing fetus have been demonstrated in laboratory animals and may include growth retardation, reduced fertility, neural tube defects, brain defects, urogenital defects, and tail defects [39, 65].

Diagnosis

Antemortem diagnosis of acute lead poisoning should be based on a history of exposure, clinical signs, and blood lead concentrations greater than 0.35 μg/ml. Hematologic abnormalities may also be indicative of lead toxicity and may include anemia, anisocytosis, poikilocytosis, polychromasia, basophilic stippling, metarubricytes, and hypochromia. Postmortem gross and histologic lesions in cattle are non-specific and therefore definitive diagnosis relies on the clinical history and liver and kidney lead concentrations [2]. Acid-fast positive lead inclusion bodies within nuclei of proximal renal tubular epithelial cells may be observed in some cases, although the presence of these inclusions is variable. Brain lesions are usually absent, but mild to moderate brain swelling may be appreciated along with vascular congestion. In select cases of subacute lead poisoning laminar cortical necrosis may be observed [66].

Treatment

In cattle, chelation with calcium disodium edetate (Ca-EDTA) is the preferred option. It may be administered

intravenously or subcutaneously at a dose of 110 mg/kg daily divided into two doses for two to three days and then repeated two days later. Thiamine may also be utilized as an adjunct to Ca-EDTA treatment in ruminants; in adult cattle a dose of 250–2500 mg/day is recommended and for calves 2 mg/kg daily. Cathartics such as magnesium sulfate (400 mg/kg per os [p.o.]) may be administered or a rumenotomy may be performed to remove larger ingested lead sources but is rarely successful in cases of particulate lead ingestion. Convulsions may be controlled by barbiturates or tranquilizers. Withdrawal times in cattle may be greater than one year and can be estimated by periodically monitoring blood lead concentrations [28].

Selenium Toxicosis

Selenium poisoning is most commonly attributed to (i) ingestion of selenium-concentrating plants grown in soils high in selenium content; (ii) accidental overdoses of selenium from ingestion of improperly mixed rations or injections; and (iii) environmental contamination due to either plant accumulation or water contamination [67]. Selenium is a non-metallic element that is an important antioxidant but can be toxic in excessive amounts. Naturally occurring selenium toxicosis is most often a regional problem in the western USA between the Rocky Mountains and the Mississippi River. Grains and forages grown in high-selenium regions may passively accumulate toxic levels of selenium, causing chronic toxicosis in herbivores [68]. The toxic dose of selenium in ruminants is unclear. References range from 2.2 to greater than 20 mg/kg body weight across species [67]. Table 69.2 lists common obligate selenium-accumulating plants and potential secondary selenium- accumulating plants.

Table 69.2 Selenium-accumulating plants of the USA.

Obligate selenium-accumulating plants
Milk vetches (*Astragalus* spp.)
Golden weeds (*Conopsis* spp.)
Woody aster (*Xylorhiza* spp.)
Princes plume (*Stanleya pinnata*)
Secondary selenium-accumulating plants
Acacia (*Acacia* spp.)
Sages (*Artemisia* spp.)
Asters (*Aster* spp.)
Saltbrush (*Atriplex* spp.)
Paintbrush (*Castilleja* spp.)
Bear tongue (*Penstemon* spp.)
Gumweed (*Grindelia* spp.)

Source: Adapted from [4].

In adult cattle clinical signs of acute selenium toxicosis may include respiratory distress, lethargy, restlessness, anorexia, watery diarrhea, pyrexia, teeth grinding, ptyalism, stilted gait, tetanic spasms, and death. Signs have been reported as early as 8–10 hours following exposure or as late as 36 hours. In cases of chronic selenium toxicosis ("alkali disease") resulting from long-term ingestion of seleniferous forages, clinical signs including depression, weakness, anorexia, weight loss, emaciation, diarrhea, anemia, hair loss, hoof deformities, and lameness may be observed [67, 68]. Reproductive abnormalities secondary to clinical and subclinical selenium toxicosis have been reported in multiple domestic species including cattle. However, a clear association between selenium toxicosis and abortion is not well documented. Reproductive abnormalities including decreased conception rates and increased fetal resorption rates were documented in cattle, sheep, and horses fed diets containing 20–50 mg selenium/kg [67].

Selenium is efficiently transferred across the placenta into fetal tissues, most notably the liver, during gestation. Excessive selenium concentration in the fetus may be teratogenic and abortifacient. Selenium levels in the fetal liver have been reported to reach as high as eight times that of the dam. Normal fetal hepatic selenium concentrations are reported to be 0.3–1.2 ppm. In a study conducted by Yaeger et al. [68] high fetal liver levels (3.47–5.25 ppm) were present in clinically healthy calves, so concentrations above 3.0 ppm are not necessarily indicative of toxicosis. Subclinical selenium may influence the immune system and pregnancy outcome [68].

Management and Treatment

Avoidance of excessive selenium exposure or ingestion is recommended for prevention of clinical disease. Once diagnosed, sources of excess selenium (forages, rations) should be removed. Effective selenium chelators are not available and therefore medical treatment relies largely on supportive care, which can be lengthy especially in cases of hoof lesions [67].

Vitamin A

Vitamin A is essential for vision, growth differentiation and proliferation of epithelial tissues, bone growth, reproduction, and fetal and embryonic development. Deficiencies in pregnant animals can result in abnormal development including disrupted organogenesis, failure of embryo growth, and resorption. Conversely, excessive vitamin A, through oversupplementation (or injection), has

been linked to reproductive failure in cattle. Though uncommon, hypervitaminosis A has been associated with fetal malformations, including neurologic and craniofacial defects with preimplantation exposure [69]. Teratogenicity appears to be increased by protein or protein-energy malnutrition [33]. Hypervitaminosis A may disrupt the retinoic acid receptor, impairing normal homeobox gene expression. Alterations in specific homeobox genes have been associated with retinoic acid-induced teratogenesis [70]. In laboratory studies using the rat as the animal model, anomalies were stage and dose dependent. When exposure occurred early following implantation, limb and genitourinary abnormalities developed [69].

Endocrine-Disrupting Compounds

Endocrine-disrupting compounds are a diverse group of compounds, natural and synthetic, that can potentially modulate or disrupt hormone homeostasis [71, 72]. Common synthetic endocrine disrupters include pesticides (DDT and organophosphorus pesticides), herbicides, fungicides, plasticizers, polystyrenes, polybrominated biphenyls and polychlorinated biphenyls (PBBs and PCBs), polychlorinated dibenzodioxins, and alkylphenolic compounds [71, 73]. These compounds are typically present in the environment at low concentrations but are persistent and have the potential to exert adverse effects on humans and animals. Exposure to endocrine disrupters may in some cases contribute to, or directly cause, infertility, pregnancy loss, intrauterine fetal death, and fetal teratogenic effects. The effects of endocrine-disrupting compounds can have impacts on developing fetuses and postnatal offspring by affecting different biochemical pathways [71]. Synthetic endocrine disrupters are suspected to have potential reproductive effects in a number of animal species, but clinical studies are limited.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a family of organic compounds with two or more fused aromatic rings. PAHs are formed from incomplete combustion of organic materials and are produced from industrial and automobile emissions, forest fires and wildfires, coal burning, volcanic eruptions, and hazardous waste sites [74, 75]. Livestock are exposed to PAHs through contaminated water, air, soil, and forages. PAH compounds are ubiquitous in the environment and are of significant concern due to their carcinogenic and other detrimental health effects demonstrated in laboratory animals and their suspected health effects in humans and domestic animals. The reproductive and developmental impacts of PAHs in domestic animals have not been thoroughly studied. PAH compounds have a steric resemblance to steroid molecules. In studies using the rat as a model, the PAH benzo(a)pyrene affected serum concentrations of progesterone, androgens, estrogens, prolactin, and, indirectly, luteinizing hormone. PAHs have also been shown to have toxic effects on rodent fetal development, resulting in fetal death, absorption, and teratogenic effects. The effect of these compounds on the reproductive health of cattle has not yet been clearly elucidated [74].

Polychlorinated Biphenyls

PCBs are chlorinated hydrocarbons that have been extensively utilized in industry in hydraulic fluids, transformer oils, plastic material, and lubricants. PCBs were widely synthesized from the 1920s to the 1970s in the USA and are still being produced in many developing countries. The chemical stability of PCBs allows these chemicals to accumulate in the environment and their lipophilic nature permits them to concentrate in fatty tissues [76]. Huge concentrations of PCBs have been found in sewage sludge from industrial and agricultural origins that is utilized as pasture and forage fertilizer. Cattle may become exposed from ingesting contaminated water and food. Although studies in large animals are scarce, PCBs have been shown to be embryotoxic in a number of species. PCBs either alone or in combination can impair normal oocyte maturation [71]. PCBs act as synthetic endocrine disrupters that affect steroid synthesis by increasing the activity of cytochrome P450 via their high affinity for estradiol and/or arylhydrocarbon receptors. Select PCBs have been shown to increase the secretion of oxytocin from the bovine ovary and $PGF_{2\alpha}$ from uterine endometrial cells. Alterations in progesterone and oxytocin induced by PCBs and other endocrine disrupters can impair normal ovulation via either luteal regression or prolonging the lifespan of the corpus luteum, but also potentially impair blastocyst implantation and recognition of pregnancy due to disturbed ratios of $PGF_{2\alpha}$ to PGE_2 [77]. In a study using the rabbit as an animal model, direct exposure of the pregnant uterus to PCBs resulted in high embryo PCB concentrations and in some cases induced embryo toxicity and pregnancy loss [76].

Organochlorine Insecticides

Organochlorine insecticides encompass a wide array of compounds that can be divided into three distinct groups including the dichlorodiphenylethanes (e.g. DDT, dicofol,

perthane, methocychlor), the hexachlorocyclohexanes (e.g. benzene hexachloride, chlordane), and the chlorinated cyclodienes (e.g. aldrin, endrin, dieldrin) [40]. The most infamous organochlorine insecticide is dichlorodiphenyltrichloroethane or DDT.

DDT is a chlorinated hydrocarbon that was widely used in the USA as an insecticide following the Second World War until it was banned in 1972 due to potential human and animal health hazards and environmental impacts. DDT is highly resistant to degradation and thereby is able to persist in the environment for long periods. DDT and other organochlorines can be absorbed orally or topically. In some developing countries persistent chlorinated hydrocarbons such as DDT, aldrin, and dieldrin (all of which are banned in the USA and Europe) are still used for insect control and can potentially contaminate cattle pastures and forages, leading to intoxication. Organochlorine insecticides exert their effects on the peripheral nerves and CNS by slowing Na^+ influx and inhibiting K^+ efflux, which causes partial depolarization of the neuron and thereby decreases the threshold for another action potential, resulting in premature depolarization of the neuron. DDT can also induce necrosis of the zona fasciculata and the zona reticularis layers of the adrenal gland, resulting in mineralocorticoid and glucocorticoid deficiency. The aryl hydrocarbons and cyclodienes decrease action potentials and also inhibit postsynaptic binding of γ-aminobutyric acid (GABA) [78].

In laboratory animal studies, organochlorines such as DDT have demonstrated estrogenic-like effects resulting in impaired fertility and pregnancy losses [40]. In a study by Mlynarczuk et al. [77] DDT along with hexachlorocyclohexanes and PCBs appeared to stimulate oxytocin synthesis in the corpus luteum of pregnant cows, decreasing the ratio of progesterone to oxytocin which presumably affected the force of myometrial contractions and increased the risk of abortions.

Clinical signs in cases of acute toxicosis in large animals may include apprehension, hypersensitivity, belligerence, muscle fasciculations of the face, neck, and limbs, and convulsions. There is no specific antidote for organochlorine poisoning; instead treatment is symptomatic. In cases of dermal exposure the animal should be washed with soap and water. Activated charcoal may be administered in cases of acute toxicity [78].

Organophosphate and Carbamate Insecticides

Livestock intoxication with most insecticides is often accidental and due to topical or oral dosage miscalculations for parasite control and formulation errors. However, intoxication may also result from the improper storage of insecticides that allows cattle access to the chemicals [79].

Organophosphates and carbamates both inhibit the activity of acetylcholinesterase by binding the enzyme and preventing it from hydrolyzing acetylcholine [80]. Inhibition of acetylcholinesterase results in the accumulation of acetylcholine, which causes overstimulation of muscarinic and nicotinic acetylcholine receptors. Toxicity is typically acute in nature with rapid onset of clinical signs (15 minutes to 1 hour). Common clinical signs in animals may include ptyalism, lacrimation, respiratory distress, muscle fasciculations and tremors, agitation, ataxia, and death [80]. Anticholinesterase compounds are potentially embryotoxic, fetotoxic, and placentotoxic due to the high concentration of acetylcholinesterase in these placental and fetal tissues. Organophosphates and carbamates readily cross the placenta and act on cholinergic and non-cholinergic components of the developing nervous system and other organs [40]. Diagnosis of suspected organophosphate or carbamate poisoning relies on quantifying the levels of acetylcholinesterase inhibition in the blood from live animals and in brain tissue (cerebral cortex preferably) from deceased animals. Inhibition of more than 70% acetylcholinesterase is considered positive for intoxication [80].

Treatment

In cases of cattle intoxication secondary to topical exposure the animal should be bathed with soap and water to remove any residual insecticide. Medical treatment is restricted to the use of atropine, activated charcoal, and supportive care. Atropine competitively inhibits acetylcholine at muscarinic receptors thereby reducing muscarinic effects. The recommended dose of atropine is 0.25–0.5 mg/kg body weight. One-fourth of the initial dose may be given intravenously and the rest subcutaneously or intramuscularly. The dose may be repeated if clinical signs recur. Dosages of activated charcoal range from 0.45 to 0.9 kg in adult cattle orally [80].

Exogenous Hormones

Therapeutic Use

Birth in the cow is triggered by an abrupt rise in fetal plasma cortisol, which induces increased synthesis of estrogens and prostaglandins. The elevation of estrogens and prostaglandins leads to regression of the corpus luteum and the rapid decline of maternal progesterone levels. The prepartum withdrawal of progesterone resulting from luteolysis appears to be essential in the calving process. Induction and

manipulation of bovine parturition via the use of exogenous (synthetic) hormones is often instituted as a management tool. Exogenous hormones, including prostaglandins, glucocorticoids, estrogens, and oxytocin, are commonly utilized to induce abortion or parturition according to the stage of pregnancy [81]. Potential reasons for termination of pregnancy or early parturition in cattle may include advancing the calving date in late-conceiving cows, synchronizing the beginning of lactation, terminating prolonged gestations, controlling fetal development in cases of oversized fetuses, treating conditions such as uterine hydrops, or where termination is necessary for the health of the cow [82].

Table 69.3 Potentially harmful xenobiotics in pregnancy.

Xenobiotic	Potential fetal effects
Acepromazine	Phenothiazines should be avoided near term due to fetal CNS depression
Albendazole	Associated with decreased conception rate (i.e. embryolethality) at a dose of 25 mg/kg in cows at 7 and/or 14 days of gestation
Aminoacetonitrile	Associated with skeletal defects in cattle
Aminoglycosides (amikacin, gentamicin)	Cross placenta. In rare cases amikacin has been associated with eighth cranial nerve toxicity or nephrotoxicity in the fetus
Atropine	May produce fetal tachycardia
Barbituates (pentobarbital, thiamylal, thiopental)	Cross placenta and induce respiratory depression; pentobarbital possibly increases neonatal mortality
Carbon monoxide	Associated with abortions and stillbirths secondary to hypoxia
Chloramphenicol	Synthetic bacteriostatic antibiotic that may decrease protein synthesis in the fetus particularly in bone marrow
Corticosteroids (cortisol, dexamethasone, prednisone, prednisolone)	Associated with fetal and neonatal articular cartilage defects Associated with increased incidence of congenital defects including cleft palate Induces parturition in cattle during late gestation
Diazepam	Associated with congenital defects in humans, rats, and mice in first trimester
Dimethylsulfoxide (DMSO)	Teratogenic in laboratory animals, not advised for use in pregnant animals
Diethylstilbestrol (DES) and estradiol	Contraindicated during pregnancy, cause fetal malformations of genitourinary system, possible fetal bone marrow depression
Griseofulvin	Teratogenic in cats and rats
Lidocaine	Probably safe but may induce fetal bradycardia
Metronidazole	Teratogenic in laboratory animals
Parbendazole	Anthelmintic associated with vertebral column and other skeletal defects in cattle
Phenothiazine	Anthelmintic associated with primary photosensitivity in cattle and a suspected cause of abortion
Prostaglandin($PGF_{2\alpha}$) cloprostenol, misoprostol)	Used for abortifacient or parturition induction in cattle
Quinolones (ciprofloxacin, enrofloxacin)	Associated with fetal cartilage defects
Sulfonamides (trimethoprim sulfa, sulfadimethoxine)	Cross the placenta, and have caused congenital defects in rats and mice but defects not reported in other species. Avoid long-acting sulfonamides due to risk of folate antagonism and bone marrow depression
Testosterone	Masculinization of female fetus
Tetracyclines (doxycycline, oxytetracycline, tetracycline)	Can induce bone and teeth malformations and dental staining in the fetus; may cause toxicity in mother
Urea and high protein diets	Associated with abortions and embryonic death in cattle
Warfarin	Causes embryotoxicity and congenital malformations (neural tube defects in laboratory animals)
Xylazine	Contraindicated in pregnant cattle due to increased tendency of premature parturition (Gilbert R)

Sources: Adapted from [3, 39, 87].

Accidental or Inappropriate Use

$PGF_{2\alpha}$ and its analogs (cloprostenol and fluprostenol) are powerful luteolytic agents that induce rapid corpus luteum regression and cessation of its secretory activity, uterine smooth muscle contraction, and cervical relaxation [83]. In cases where induced abortion is desired, $PGF_{2\alpha}$ and its analogs may be administered. Administration of $PGF_{2\alpha}$ 5–7 days following ovulation and up to 150 days of gestation is typically effective for termination of pregnancy. Estrogens administered within 72 hours of ovulation can impair oviductal embryo transit, thereby preventing embryo implantation. After this period, estrogens induce luteolysis, thereby inducing abortion.

A combination of $PGF_{2\alpha}$ and dexamethasone/flumethasone is commonly used to induce abortion between 150 and 180 days (5–8 months) of gestation but is also effective in all stages of gestation. The combination of these two hormones allows the elimination of both ovarian corpus luteum progesterone and placental progesterone production. Following administration, abortions will usually occur within five days. During the last month of gestation dexamethasone or prostaglandins may be used alone to induce parturition [84].

Oxytocin is a nonapeptide hormone produced by neurons in the paraventricular or supraoptic nucleus of the hypothalamus and stored in the pars nervosa of the pituitary gland [85]. During parturition and lactation, and in response to osmotic challenge, oxytocin is released into the systemic circulation from axon terminals in the neurohypophysis and acts directly on the smooth muscle of the uterus to induce rhythmic contractions. Exogenous oxytocin is often used as a uterine contractor to precipitate and accelerate normal parturition and postpartum evacuation of retained placentas or uterine debris. It also stimulates contraction of smooth muscle cells of the mammary gland for milk let-down and is used in cases of agalactia [83].The responsiveness of uterine musculature to oxytocin varies significantly with the stage of the reproductive cycle. Oxytocin can result in luteolysis in the cycling cow or heifer by inducing the release of $PGF_{2\alpha}$ from endometrial cells. During early phases of pregnancy, the uterus is thought to be relatively insensitive to the effects of oxytocin, while in the late phases the sensitivity is markedly increased. However, in a study by Yildiz and Erisir [86] oxytocin administration to pregnant cows four to seven days following insemination resulted in a higher risk of pregnancy loss and early embryonic mortality.

Pharmaceuticals

The administration of certain pharmaceuticals to pregnant animals can pose a significant risk to the developing embryo and/or fetus. Before any medication is administered to a pregnant animal, the drug label should be closely reviewed to ensure that the product is safe for prenatal use. Table 69.3 lists some contraindicated xenobiotics along with their potential fetal effects. However, it should be noted that many of the effects have been documented only in laboratory animals.

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Heritable Congenital Defects

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Introduction

Heritable congenital defects are being recognized at an increasing rate, but only a few are unequivocally propagated as a result of specific but unrelated trait selection. In some cattle populations, inherited defects have become a frequent and economically important source of pregnancy loss. Veterinarians, animal scientists, and cattle breeders should be aware of heritable defects and prepared to investigate and report animals exhibiting abnormal phenotypes.

Genetic testing has changed cattle production, not only in terms of traits beneficial to production, but also in the ability to identify and manage harmful genetic defects. As selection practices amplify the genetic impact of certain individuals, the potential for observation of heritable defects increases concordant with increases in the frequency of deleterious alleles. The surveillance of such disorders has become a part of bovine health programs.

Congenital abnormalities are very often discounted as randomly occurring "accidents of gestation;" the defects may not be deemed reportable and appropriate samples may not be collected. Failure of identification or delay in detection of heritable defects may allow further distribution of the causative alleles. Obvious defects such as skeletal malformations, extensive soft tissue abnormalities, severe neurological disorders, and diseases of the skin are more likely to be recognized, whereas defects involving internal organs or abortions and stillbirths may be less obvious and more easily missed. Surveillance may be further compromised by the reluctance to report potentially heritable disorders. The major cattle breed associations of the USA and Europe have staff assigned to receive reports of suspect inherited deficits and often coordinate or otherwise facilitate investigations.

Several reviews on inherited disorders in cattle have been published [1–3], and a regularly updated electronic database, Online Mendelian Inheritance in Animals (OMIA), is available at www.omia.org [4].

This chapter describes the fundamentals of report investigations, and morphologic characteristics, mode of inheritance, breeds affected, and availability of specific tests or anatomic hallmarks for select heritable bovine fetal abnormalities. A comprehensive summary of all known heritable congenital abnormalities is beyond the scope of this chapter. The purpose of this review is to emphasize heritable bovine fetal abnormalities frequently encountered, recently described, active in current cattle populations, and those for which the mutation has been identified and a test is available.

Event Investigation

The etiologies of congenital defects in cattle can be divided into heritable, toxic, nutritional, and infectious categories; distinguishing among different causes is essential in the management of the disease but may be challenging. Reports of congenital defects by cattle breeders require at least a cursory epidemiologic investigation when the animal's phenotype does not immediately identify to a known characterized defect. Given the economics and speed with which genome sequencing can be done, the opportunities to advance knowledge and provide new tools to assist breeders have never been more feasible. Samples for possible DNA extraction must be preserved at the start of investigation and can be frozen (tissue) or chilled (whole blood) until the need to process has been determined.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Generally, it is advisable to collect samples from an affected calf for DNA, and from the presumed sire and dam if easily available and before they are removed from the herd. Samples related to affected calves resulting from artificial insemination can be obtained later from stored semen and may already have some genomic data on file.

The minimum database must include examination of the affected animal(s) with a good characterization of the phenotype (ideally a necropsy performed by a pathologist) and a thorough clinical history. It is seldom possible on the basis of clinical features alone to be confident of the etiology, but information such as the pedigree details, knowledge of the number of animals affected, and maternal diet during pregnancy may provide useful clues. Additionally, infection of dams with teratogenic viruses during pregnancy may result in congenital abnormalities. As a result, knowledge of individual and herd immunization and incidence of relevant viral infections must be determined. In some cases, teratogenic or nutritional causes of congenital abnormalities may appear very similar to genetic causes. Therefore assessment of historical data is essential in any attempt to distinguish genetic and acquired causes of congenital defects. Not only a current clinical history but also a record of environments and movement of animals and contemporaries throughout gestation is needed, as many teratogenic effects are initiated long before parturition.

When the pedigree of the sire and dam are known the corresponding breed association is an excellent resource. Breed associations have access to extended pedigrees available on the internet (usually under expected progeny differences [EPD]) where you can look for ancestral relationships expected in recessive traits. The pedigrees also often list the genotyping that has been done regarding carrier status of well-characterized defects. When the parents are registered there may be support for investigating the defect as part of the breed's improvement or quality assurance program.

For further guidance, a comprehensive and thorough review of the approach to investigate congenital skeletal diseases in farm animals, with emphasis placed on investigating genetic causes, is available [5].

Arthrogryposis Multiplex

Arthrogryposis multiplex (AM) is a lethal autosomal recessive genetic defect that originated in Angus cattle. Beginning in 2008, researchers in collaboration with the American Angus Association (AAA) investigated abnormal calves believed to fit the description of what was then called AM and commonly referred to as "curly calf syndrome" in Angus cattle. Within two months, researchers obtained samples and pedigrees from affected calves and their parents, the mutation was identified, the DNA test was developed and validated, and the status of over 700 AI bulls was determined [6].

Calves with AM are born dead or die shortly after birth. They are small for their gestational age and have markedly diminished muscle mass, but dystocia is common as a result of the congenital arthrogryposis, scoliosis, and torticollis. It appears that in AM an essential protein that allows communication between nerves and muscle tissue is absent, so the calf (which fails to move in utero) is born with the joints of all four limbs fixed and the legs twisted. There are several characteristics of AM, including arthrogryposis, kyphoscoliosis, and decreased muscling [7] (Figure 70.1).

The mutation is a deletion that involves three genes; one of these genes is involved in the development of nerve and muscle. Affected calves are missing about 23 kb. These missing base pairs result in complete loss of function of all three genes in homozygous calves [6].

Bovine Dwarfism

Dwarfism as a heritable condition has been reported in many mammals, including multiple breeds of cattle [1, 8–14]. Several types of inherited chondrodysplasia have been reported in the bovine. Although the phenotypic expression is variable, all are characterized by systemic skeletal disorders, including shortness and deformity of limbs, head, and vertebrae. Dwarfism has been recognized in the Angus, Brown Swiss, Danish Red, Dexter, Hereford, Holstein, Japanese Brown, and Shorthorn breeds [15]. No single gene or mutation



Figure 70.1 AM in an Angus calf that was born dead. Note generalized muscle hypoplasia, contracted forelimbs, and fixed hindlimbs. *Source:* Image courtesy of Dr. David Steffen, University of Nebraska.

is responsible for all reported cases of bovine dwarfism. Here we describe two unique forms of bovine dwarfism.

Dexter bulldog dwarfism has been a major problem for the Dexter breed since its description in the early nineteenth century [16]. Homozygosity for the Dexter dwarf mutation is lethal and fetuses are generally aborted at approximately seven months of gestation. Clinically, the defective fetuses are characterized by an extreme disproportionate dwarfism, a short vertebral column, marked micromelia, large abdominal hernia, and relatively large head with a retruded muzzle, cleft palate, and protruding tongue [17].

Bulldog dwarfism of Dexter cattle is caused by two discrete mutations in the aggrecan (*ACAN*) gene, and DNA testing is available for the mutation [17]. When defined strictly on the lethal bulldog phenotype, the inheritance pattern of Dexter dwarfism is described as autosomal recessive (i.e. the bulldog dwarf is homozygous for the genetic mutation). However, because heterozygotes have an intermediate dwarf phenotype, the trait can also be described as having an incomplete dominance mode of inheritance (8% expression of *ACAN* in heterozygotes compared with homozygous normal) [17]. This has presented a major problem for Dexter breeders, because the favored Dexter phenotype, featuring short legs (a mild form of disproportionate dwarfism), tends to be heterozygous for the bulldog mutation. Selecting for the favored Dexter phenotype maintains a lethal allele at a high frequency [17].

Dwarfism in Aberdeen Angus calves was described in 1951 and determined to be autosomal recessive [10]. The AAA handled the issue by the virtual annihilation of all animals associated with the primary source herd [18]. Following the efforts of the breed association to eliminate dwarfism, there were no certified reports to the AAA of dwarfism in registered Angus cattle from the 1970s until 2002. At the start of the twenty-first century, abnormal Angus calves were reported in several herds in the western USA. Unlike the previous form of dwarfism, these calves appeared normal at birth, but failed to grow and after several months appeared to have abnormally short legs and thick bodies. Gross and histopathological examination of these calves indicated evidence for diminished endochondral ossification and other features consistent with dwarfism, including the protrusion of the alar wing of the basisphenoid bone into the cranial cavity, abnormalities of the ventral vertebral bodies, and curving of the transverse vertebral processes ("long-nosed" dwarfism) [19]. The genetic defect of the long-nosed Angus dwarf is not the same as the defect in Dexter (mutations in *ACAN*) or Japanese brown cattle (*LIMBIN* mutations). A non-sense mutation of cGMP-dependent type II protein kinase (*PRKG2*) leading to a premature stop codon and 85 C-terminal amino acids being truncated, including a large portion of the kinase domain, is the causative mutation for Angus dwarfism. Growth plate organization is regulated, in part, by PRKG2 regulation of sex-determining region Y box 9 (SOX9) phosphorylation. Phosphorylation of SOX9 is required for proper transition of chondrocytes from a proliferative to hypertrophic growth phase. Therefore dysfunction within the growth plate chondrocytes results in abnormal long bone growth and dwarfism in Angus cattle [19, 20].

Brachyspina Syndrome

Brachyspina syndrome is a lethal malformation in the Holstein breed [21–23]. The syndrome is characterized by calves born dead following a slightly prolonged gestation. The gross morphology of brachyspina syndrome shares many features with short spinal lethal syndrome in cattle, first described in Old Norwegian Mountain calves born in 1930 [24]. Brachyspina calves are growth retarded, with severe shortening of the entire vertebral column and limbs. Most vertebral segments have some lack of organization, with irregular ossification separated by cores of cartilage that prevent identification of individual vertebra. Multiple defects of the internal organs, including renal dysplasia and intestinal atresia, are consistently present, whereas brachygnathism and caudal dislocation and compression of the brain are inconsistently present. The appendicular skeleton may or may not be affected.

Although variation in morphology caused by a common syndrome is not unusual, six reported cases of brachyspina syndrome may have had more than one etiology, since they were morphologically diverse. In two reports of brachyspina syndrome, genealogical examination showed that the cases occurred in familial patterns [22, 23], a common ancestor to all parents was found, and the occurrence of the abnormal calves could potentially be explained by transmission of recessive alleles. Although these observations supported the hypothesis of a genetic basis of brachyspina syndrome, association does not prove causation. Widely used sires may occur as common ancestors in the pedigree of malformed calves without that individual being a carrier, or without the defect having an inherited etiology. If brachyspina syndrome is inherited as an autosomal recessive anomaly, it may be an emerging worldwide disease in the Holstein breed.

Complex Vertebral Malformation

Complex vertebral malformation (CVM) syndrome is a recessively inherited lethal disorder in the Holstein breed that increases embryonic death, abortion, and perinatal

death. CVM fetuses have a composite of phenotypic abnormalities, including axial skeletal deformities (e.g. hemishaped and misshaped vertebra, ankylosis of mainly the cervicothoracic vertebra, and scoliosis), symmetric arthrogryposis of the lower limbs, craniofacial dysmorphism, and cardiac anomalies [15, 25, 26]. Clinical heterogeneity among affected calves may make it difficult to make a diagnosis of CVM; however, a presumptive diagnosis can be made at necropsy (if pedigree information is available).

Genealogical research identified Carlin-M Ivanhoe Bell (registration number 1667366), an elite Holstein-Friesian bull born in 1974, to be the main ancestor of cattle carrying this mutation [15]. Because of the superior lactation performance of his daughters, Ivanhoe Bell was extensively used for two decades. Carriers of the CVM mutation exist in Holstein cattle populations worldwide and the frequency of CVM carriers among Holstein sires has reached an alarming level [25, 27–30].

By comparing DNA sequences of unaffected and affected calves, research [31] has uncovered a point mutation in the form of a transversion (G→T) in the *SLC35A3* gene, causing a valine at position 180 to be replaced with phenylalanine. The *SLC35A3* gene product is a Golgi-resident transporter critical for the formation of glycoproteins and, ultimately, axial skeleton development. A screening test (DNA) for CVM is now available and has been widely performed on Holstein sires. Pedigree information now includes the designation "TV" for tested animals free from CVM and "CV" for carriers of CVM.

Congenital Contractural Arachnodactyly

Congenital contractural arachnodactyly (CA), or "fawn calf syndrome," is a non-lethal autosomal recessive genetic defect of Angus cattle. CA calves are normally born alive and most can walk, suckle, and survive. The birthweight of CA calves is "normal." The phenotype is subtle, so that CA may not initially be recognized as a defect. CA is a developmental defect involving reduced elasticity of the connective tissue of muscles first identified in Victoria, Australia in 1998 but now reported in many countries [32]. Although CA is a less severe disease than lethal genetic defects of Angus calves, without human intervention up to 20% of CA calves die soon after birth because they are unable to stand and suckle [32]. CA manifests in newborn calves as elongated limbs, congenital proximal limb contracture, congenital distal limb hyperextension, and congenital kyphosis, with significant postnatal improvement in these clinical signs as the calf grows and matures [32].

Researchers have identified the gene that causes CA and have characterized the specific mutation responsible for CA as a deletion of at least 54 kb, severely compromising function [33]. A diagnostic assay has been routinely used since 2011; however, the assay should not be used within Gelbvieh cattle due to the known presence of false positives. The false positives are due to a yet uncharacterized allele within specific Gelbvieh pedigrees.

Developmental Duplication

In 2012, a rising incidence of notomelia (polymelia where the extra limb is rooted along or near the midline of the back; Figure 70.2) and other forms of polymelia was noted in cattle of the Angus breed in Australia and the USA [34]. In 2013, a single base pair substitution was identified in the *NHLRC2* gene of these bovine polymelia cases. The mutation causes valine-to-alanine substitutions at residue 311 (V311A) of the NHLRC2 protein [35]. The recessive syndrome of diverse congenital malformation phenotype in cattle caused by *NHLRC2* mutation is called "developmental duplications (DDs)" [34].

Homozygosity of the *NHLRC2* mutation in these calves is associated with several different forms of polymelia (cephalomelia, notomelia, and pygomelia [accessory hind limbs]) producing both accessory forelimbs and accessory hindlimbs as well as conjoined twinning (Figure 70.3) (including parasitic rachipagus [fusion of the back] and caudal duplication syndrome). In a 2013 survey of Angus cattle, calves were identified with neural tube defect-related lesions that included but were not limited to Chiari malformation, dermal cyst, encephalocele, meningocele, spina bifida, polymelia and conjoined twinning. Although the allele prevalence of this V311A *NHLRC2* mutation in the registered Angus population was approximately 16% at



Figure 70.2 Developmental duplication in an Angus calf. Note the notomelia. *Source:* Image courtesy of Dr. Brian Whitlock, University of Tennessee.



Figure 70.3 Developmental duplication in Angus calves born dead. Note the parasitic rachipagus (fusion of the back). *Source:* Image courtesy of Dr. Amy Weatherly, Wolf River Veterinary Services, Grand Junction, Tennessee.

the time, 11 of 13 consecutive cases with neural tube defect-related lesions were homozygous for the *NHLRC2* mutation, demonstrating a causal association [36].

Polymelia is, however, rare, even in Angus calves that are homozygous for this *NHLRC2* mutation. Population genotype ratios have shown that almost half of the expected number of *NHLRC2* mutant homozygotes is present in the Angus population as phenotypically normal individuals and the other half is missing from the population, apparently due to various phenotypes, testing of those animals, and possibly a high rate of early embryonic death (J. Beever, personal communication) [37]. The incidence of newborn calves with the various malformations associated with homozygosity of this *NHLRC2* mutation, including polymelia, is not sufficient to explain the number of homozygotes missing from the population. The incomplete penetrance of developmental defects in bovine conceptuses homozygous for the *NHLRC2* mutation suggests that there are additional genetic or environmental determinants of the resulting phenotype and that the newborn calves with polymelia and other neural tube defect-related lesions observed in this DD syndrome are rare survivors of what are normally lethal embryonic malformations caused by *NHLRC2* mutation.

Hypotrichosis

Congenital hypotrichosis is partial to complete lack of hair that is obvious at birth or within the first weeks of life. Affected calves are often born with very short, fine, kinky hair that may fall out, leaving bare spots. As a result, affected calves may suffer from cold stress, leading to death or failure to thrive. Congenital hypotrichosis can have a non-genetic or genetic etiology. In cattle, non-genetic hypotrichosis can be caused by iodine deficiency, adenohypophyseal hypoplasia, maternal ingestion of European white hellebore (*Veratrum album*), intrauterine infection with bovine viral diarrhea virus, or autoimmunity.

Various forms of genetic hypotrichosis have been described in serval cattle breeds including Hereford, where it is inherited as an autosomal recessive trait [38]. More recently, congenital hypotrichosis has been reported in Belted Galloway cattle [39]. The inherited hairless phenotype can vary in severity and is frequently observed on the legs and head, with affected calves generally being identified at birth or shortly thereafter. The inherited condition may vary in expression as affected animals mature and may be less noticeable in older animals, with the hair coat color sometime appearing "frosted" and the tail switch may be underdeveloped. Some affected animals may also have reduced growth rates. Both single locus and haplotype whole genome association analysis determined that single-nucleotide polymorphisms (SNPs) located on bovine chromosome 29 were significantly associated with congenital hypotrichosis phenotype in cattle. Homozygosity analysis revealed a critical region spanning approximately 770 kb at the top of bovine chromosome 29. Possible candidate genes were sequenced to determine the causative mutation. Sequence analysis revealed an A1684T substitution in exon 9 of hephaestin-like 1 resulting in a premature stop codon. Subsequent genotyping showed all affected calves were homozygous for the point mutation. Hephaestin-like 1 is responsible for copper ion transport. Copper deficiency has been shown to cause anemia, poor

immune function, slower growth rates, and discolored or poor hair coats in cattle. More than 300 animals have been tested, with no phenotypically normal animals exhibiting the homozygous mutant genotype [39].

A small deletion mutation was found in the bovine *KRT71* gene that is associated with the congenital hypotrichosis phenotype in Hereford cattle. The K71 protein is a hair follicle-specific protein that is thought to participate in the formation of structurally important keratin intermediate filaments within the inner root sheath of the hair follicle. The mutation is predicted to result in a premature stop codon that will produce either an mRNA transcript that is degraded or a truncated protein that is unable to participate in proper heterodimer formation in the inner root sheath of the hair follicle. It is expected that formation of the keratin intermediate filaments, necessary for structural support, and hair shaft molding do not occur. Homozygosity for a loss-of-function mutation in bovine KRT71 is associated with abnormal hair development, corresponding to congenital hypotrichosis in Hereford cattle (J. Beever, personal communication).

Inherited Congenital Myoclonus

Inherited congenital myoclonus (ICM), also known as idiopathic epilepsy, is a seizure disorder observed in Hereford cattle and their crosses caused by an autosomal recessive genetic defect incompatible with life. ICM is characterized by spontaneous and stimulus-responsive myoclonic spasms that are prenatal and prevent calves from rising at birth. Affected calves have a "normal" phenotype when they are not experiencing seizures. Environmental stressors (heat, cold, weaning) can trigger the seizures, and the seizures can last from minutes to more than one hour. Bovine ICM has been attributed to a severe disturbance of glycine-mediated neurotransmission in the spinal cord [40, 41]. The observed phenotype is due to a non-sense mutation in codon 24 of the glycine receptor polypeptide, which results in truncation of the α_1-subunit and subsequent loss of cell-surface expression [42].

Junctional Epidermolysis Bullosa

Hereditary junctional epidermolysis bullosa (JEB) is a recessive inherited blistering disorder of the skin and mucous membrane in which tissue separation occurs within the lamina lucida (i.e. under the basal plasma membrane of the basal keratinocytes and above the basement membrane) of the basement membrane zone at the dermal–epidermal junction. Congenital JEB has been sporadically observed in Charolais cattle, but the causative mutation had not been identified until recently. Four cases that were born in the same French herd from consanguineous matings were recently reported [43]. Analysis of the pedigree data revealed that all JEB-affected animals traced back, on both maternal and paternal sides, to a single founder bull born in the UK in 1986, which suggested an autosomal recessive mode of inheritance as the most parsimonious model.

At birth, acral clinical lesions were observed with dysungulation of the four hooves and erosion and ulcers on the skin from the carpal and tarsal joints, fetlocks, ears, eyes, and muzzle, and in the oral cavity, most cases having ear deformities (atrophied pinna and closed ears) as well [43]. For humane reasons, as a result of anorexia, apathy, emaciation, and obvious pain, affected calves were euthanized not long after being born. Because of the rare occurrence of JEB in Charolais cattle and the rapid euthanasia of affected animals, only one case was available for DNA sampling, thus preventing the use of a classical autozygosity mapping approach. As a consequence, whole genome sequencing of this animal was completed. Ultimately, a 2831-bp deletion encompassing exons 17–23 of the integrin beta 4 (*ITGB4*) gene, a gene previously associated with the same condition in humans [44], was identified as the cause for JEB in this herd of Charolais cattle. ITGB4 encodes the beta subunit of the alpha 6 beta 4 integrin heterodimer. This transmembrane receptor is a key component of the hemidesmosome anchoring complex which connects basal keratinocytes to the basement membrane. Genotyping a second case and six parents of affected calves revealed a perfect association between this mutation and the assumed genotypes of the individuals. Analysis of genotyping data from 6870 Charolais cattle detected only 44 heterozygous animals for a 5.6-Mb haplotype around ITGB4 that was shared with the carriers of the mutation. Genotyping of 16 of these 44 animals revealed that none was a carrier of the deletion, suggesting a rather recent origin for this mutation in the Charolais breed [43]. At about the same time a similar report was madFe regarding a Charolais calf from Germany [45]. Genetic analysis revealed an *ITGB4* loss-of-function mutation where the affected calf was homozygous for a 4.4-kb deletion involving exons 17–22. Along with the dam, some Charolais cattle belonging to the affected herd carried a single copy of the deletion [45].

Maple Syrup Urine Disease

Maple syrup urine disease (MSUD) is an autosomal recessive disease of branched-chain amino acid metabolism that is caused by mutations in one or more of the four subunits of the branched-chain α-ketoacid dehydrogenase (BCKDH)

complex. In cattle, MSUD was first observed in Australia affecting both Hereford [46] and Shorthorn calves [47]. MSUD was subsequently detected in Canadian Hereford cattle in 1987 [48]. Two different mutations in the E1α subunit (branched-chain ketoacid dehydrogenase E1, alpha polypeptide [BCKDHA]) of the enzyme were later identified [49, 50]. In Herefords, a transition mutation within exon 2 (c.154C > T) of the BCKDHA gene results in a nonsense substitution (p.Q52X) causing the complete absence of E1α protein [49]. A missense substitution (p.P372L) caused by a transition in exon 9 (c.1372C > T) is responsible for MSUD in Shorthorns [50].

Although MSUD was recognized in the mid-1980s, there were very few calves reported due to negative selection based on pedigree and the implementation of molecular diagnostic testing, particularly within Australian Herefords. However, more recently several affected calves have been reported in both Herefords [51] and Black Herefords in the USA. The Hereford c. 154C > T mutation was shown to be responsible in reported cases (J. Beever, personal communication) [51]. In Black Herefords further investigation revealed a carrier frequency of approximately 13% among 287 animals tested. Furthermore, it was determined the founding sire within the Black Hereford registry was an MSUD carrier, as was his registered Hereford sire. Examination of more than 33 000 Hereford animals that had been genotyped for the c. 154C > T mutation demonstrated an allele frequency of 0.04%. However, three prominent modern AI sires were also shown to be carriers, increasing the risk of observing MSUD-affected calves over the next several calving seasons.

MSUD-affected calves are frequently born asymptomatic but rapidly develop significant neurologic symptoms within 12–24 hours following birth [46, 48, 52]. Calves appear lethargic and are unable to rise by themselves. Calves are often found laterally recumbent with occasional limb paddling or tremors followed by periodic opisthotonus. Euthanasia is often required within 48–72 hours. Field diagnosis can be made by the presence of darkish-brown urine having a burnt maple syrup-like odor. Clinical diagnosis can be made by significantly elevated serum branched chain amino acid (BCAA) levels.

Neuropathic Hydrocephalus

Neuropathic hydrocephalus ("water head") is a lethal autosomal recessive genetic defect of Angus cattle. At the same time that AM calves were being submitted, calves with hydrocephalus were also submitted. These calves were similar in description and pedigree to calves described by Dr. Denholm in Australia.



Figure 70.4 Neuropathic hydrocephalus in an Angus calf that was born dead. Note the markedly enlarged cranium. *Source:* Image courtesy of Dr. Kristie Steuer, Animal Health Center, Laramie, Wyoming.

Affected calves are born near term and weigh 10–20 kg at birth. The head is markedly enlarged (Figure 70.4). The bones of the skull are malformed and appear as loosely organized bony plates that fall apart when the head is opened. The cranium is filled with fluid and no recognizable brain tissue is evident. The spinal canal is also dilated and no observable spinal tissue is found [53]. Because of the hydrocephalic cranial enlargement, dystocia is almost invariable. Delivery per vagina can normally be achieved by incising the cephalic vesicle of the domed cranium to drain the fluid. Manually removing the skull bones from the collapsed vesicle before applying traction may be necessary.

The genetic mutation leads to the abnormal function of a protein involved in the development and maintenance of the central nervous system and results in neuropathic hydrocephalus [53]. Nearly 10% of AI sires representing a broad cross-section of registered Angus genetics were found to be carriers of this syndrome [54]. Given the number of calves reported, this frequency is higher than expected. This phenomenon could be explained by a relatively high percentage (50–70%) of pregnancy wastage in embryos and/or fetuses. This is consistent with what is known about mutations in this gene for other species (i.e. complete disruption of this gene in mice results in 100% fetal mortality before the halfway point of gestation) [55].

Oculocutaneous Hypopigmentation

From 2012 to 2014, the American Simmental Association (ASA) received reports of cattle with "white-colored" eyes and a diluted hair coat (Figure 70.5). Tissue samples

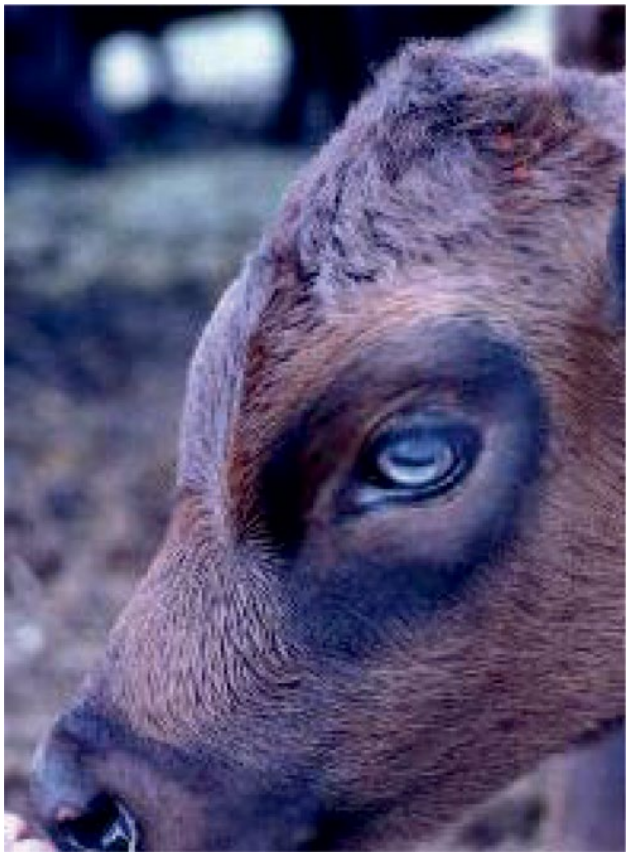


Figure 70.5 Oculocutaneous hypopigmentation in an Angus calf. Note the pale blue iris and diluted hair coat color. *Source:* Image courtesy of the American Angus Association, St. Joseph, Missouri.

collected from four affected calves were genotyped. The resulting genotypes were analyzed in contrast to the genotypes of ~80 Simmental sires. This analysis provided clear evidence that the condition was inherited as a recessive trait. Based on these results, the DNA sequence for several genes was analyzed in each of the affected calves. Within one of these genes, a mutation was identified that is predicted to impair the function of the encoded protein. An amino acid exchange in the Ras-related protein Rab-38 gene was identified as the disease causing mutation (J. Beever, personal communication) [56]. In mice, mutations within the same gene cause a very similar condition that is referred to as "chocolate," where black mice have a diluted coat color and beige-colored irises [57]. Information contained in reports and literature from the 1970s and 1980s, and in these current Simmental cases, indicate that this abnormal phenotype has little or no effect on the viability or performance of affected individuals.

Further investigation indicated the mutation was present at a relatively low frequency in the Simmental population, consistent with the very low frequency of affected calves reported over the three-year period. Examination of carrier pedigrees revealed a single Simmental bull as the most popular recent ancestor with DNA available for testing. However, several of the genotyped carriers did not have this sire in their pedigrees, indicating the mutation could have been introduced to the Simmental population much earlier. Considering this information and the prior description of similar traits in other breeds, namely heterochromia irides (HI) in Angus cattle, the possible origin of this mutation was investigated by obtaining samples from known HI carriers. Although there are very few DNA samples available from these older animals, a sample was obtained for one of the relevant Angus sires and he was found to be a carrier of this newly identified mutation in Simmental cattle. Therefore it is most likely that the mutation was introduced into the Simmental population by the use of Angus cattle during the development of black purebreds. The subsequent screening of more than 1200 Angus sires indicates the mutation has most likely been eliminated from the current Angus population via pedigree selection in the early 1980s (J. Beever, personal communication) [56].

Osteopetrosis

Osteopetrosis ("marble bone") is a lethal autosomal recessive genetic defect previously identified in humans and animal species. Cattle breeds known to be affected are Black and Red Angus, Hereford, Simmental, and Holstein. The defect was most recently reported in the Red Angus breed [58]. Calves affected with osteopetrosis are born 10–30 days early. They usually have head abnormalities that consist of brachygnathia inferior, impacted molars, and a protruding tongue. The long bones are shorter than normal, and the marrow cavities are filled with unresorbed bone (primary spongiosa) but are very fragile and can be easily broken [58].

The disease is caused by a deletion of *SLC4A2*, a gene necessary for bone remodeling during development. *SLC4A2* encodes an anion exchanger protein necessary for proper osteoclast function. Loss of *SLC4A2* function appears to induce premature cell death and results in cytoplasmic alkalization of osteoclasts, which in turn may disrupt acidification of resorption lacunae. Histological examination of affected tissues reveals scarce, morphologically abnormal osteoclasts displaying evidence of apoptosis [59].

Pulmonary Hypoplasia with Anasarca

Pulmonary hypoplasia with anasarca (PHA) is a lethal autosomal recessive disorder of a single missense mutation characterized most notably by dystocia, profound anasarca,

death of the calf, and not uncommonly death or morbidity of the cow [54, 59]. Originally, PHA calves were identified as Maine-Anjou, Shorthorn, or their crosses (composite cattle); however, cases are also reported in Australian Dexter, Hereford, and belted Galloway cattle [3, 54, 60].

Breeding of two PHA carriers would theoretically produce 25% affected fetuses. However, there appears to be a high percentage of pregnancy wastage from approximately 90–200 days of gestation. Homozygosity for the PHA mutation is lethal and fetuses are generally aborted at approximately seven months of gestation. When PHA fetuses survive to near term, they invariably result in dystocia. It appears that PHA calves thought to be "bred to the AI date" are usually premature births of calves conceived due to natural service following an AI program.

The most obvious external characteristic of PHA calves is marked anasarca, resulting in large calves (Figure 70.6), frequently requiring cesarean section. Furthermore, PHA is characterized by the absence or near absence of lung tissue (pulmonary hypoplasia; Figure 70.7), lymphatic agenesis (absence of lymph duct and nodes), and athymia [60, 61].

Heterozygous PHA carriers have a "normal" phenotype and can be identified only by genetic testing. Following identification of the gene in late 2006, over 40 popular Maine-Anjou, Shorthorn, and "club calf" sires were identified as PHA carriers. Interestingly, several of the most popular Shorthorn bulls are carriers for both tibial hemimelia and PHA. A syndrome similar to PHA was identified in Dexter cattle in 2006 when Windsor et al. [54] reported three cases of PHA in the Dexter breed. The cows were related and inheritance was felt to be autosomal recessive. Although the mutation in the Dexter breed involves the same gene as the condition seen in Maine cattle, it is not identical. The Maine-Anjou mutation was a simple point mutation, whereas the Dexter mutation was an 84-bp deletion involving the same exon as the Maine mutation (J. Beever, personal communication).



Figure 70.6 Calf with pulmonary hypoplasia with anasarca syndrome delivered by partial fetotomy. Note the severe anasarca. *Source:* Image courtesy of Dr. Brian K. Whitlock, University of Tennessee.

Recessive Defects Discovered from the Absence of Homozygous Haplotypes

Lethal recessive defects were often discovered in the past from reports of abnormal calves and subsequent breeding trials to confirm the inheritance. Defects that affect embryo loss were sometimes discovered from occasional abnormal calves that reached late gestation or parturition [21, 25] or from physiological differences between normal and heterozygous phenotypes [62]. Instead of relying on breeder reports of abnormalities, embryonic defects may more easily be discovered using fertility traits and genotypes stored in national or international databases [63].

Genotypes and haplotypes for large numbers of individuals provide a new tool for identifying lethal recessive genes. Defects that cause embryo loss are difficult to detect without genomic data, even with very large sets of phenotypic and pedigree data, because of insufficient observations per estimated interaction [64]. With genomic data, lethal recessives may be discovered from haplotypes that are common in the population but are never homozygous in live animals. This method requires genotypes only from apparently normal individuals and not from affected embryos and therefore is the opposite of most previous strategies [65]. The new approach can discover lethal defects without phenotypic data. If the number of genotyped individuals is large, the expected number of homozygous haplotypes is sufficiently large so that their complete absence is unlikely to be attributed to chance.

Five new recessive defects have been discovered in Holstein, Jersey, and Brown Swiss breeds by examining

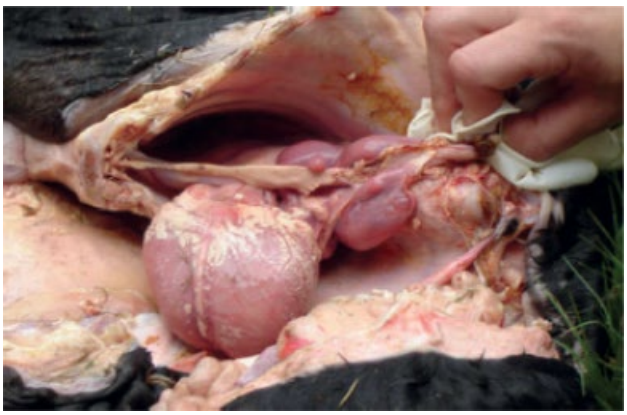


Figure 70.7 Thorax of a calf with pulmonary hypoplasia with anasarca syndrome. While the lungs of a "normal" bovine fetus at a similar stage of gestation are proportionate to those of a neonate, note the severe pulmonary hypoplasia (severely hypoplastic lungs are to the right of the heart). *Source:* Image courtesy of Dr. Brian Whitlock, University of Tennessee.

haplotypes that had high population frequency but which were never homozygous. Genotypes from the BovineSNP50 BeadChip (Illumina, San Diego, CA) were examined for 58 453 Holsteins, 5288 Jerseys, and 1991 Brown Swiss apparently normal individuals with genotypes in the US database. Phenotypic effects were confirmed using records for conception rate in a smaller population of cows from the affected breeds. Estimated effects on conception rate ranged from −3.0 to −3.7 percentage points, which was slightly larger than estimated effects for previously known lethal alleles of −2.5 percentage points for brachyspina and −2.9 percentage points for CVM [66]. Estimated effect of carrier interaction for stillbirth rate was small (≤1.8 percentage points) but comparable to the effect of CVM (1.4 percentage points) [66]. For each of the five new defects, 2.7–20.7% of elite animals in the current population are carriers. However, selection against carriers would improve fertility only slightly. For example, conception rates would increase by <1 percentage point if any of the haplotypes were eliminated from the population [66].

Tibial Hemimelia

The tibial hemimelia (TH) syndrome was first described in 1951 in Scotland in the Galloway breed of cattle [67]. This lethal condition is characterized by multiple congenital skeletal deformities, most notably shortened or absent tibia, abdominal hernia, cryptorchidism, failed paramesonephric duct development, hirsutism, and improper neural tube closure resulting in meningocele. Calves are born dead or die shortly after birth. The Scottish Galloway Association used test breeding and pregnancy termination to identify carriers and eliminate the autosomal recessive defect from Galloway breeding stock [68].

The syndrome was first described in the USA in 1974 [69], and subsequently in a case report of a female Simmental calf with arthrogryposis, ventral abdominal hernia, TH, and non-union of the paramesonephric ducts, with the suggestion that it might be a genetic disorder [70]. A decade later, TH was identified in six genetically related registered Shorthorn calves, three from the USA and three from Canada [71]. Although the skeletal abnormalities varied, the combination of TH, abdominal hernia, and meningocele was felt to be strikingly similar to the condition previously described and determined to be inherited as an autosomal recessive trait in Galloway cattle. Pedigree analysis of the Shorthorn calves suggested that homozygosity of an autosomal recessive allele was responsible for the defect.

Identification of the defective gene mutation causing TH was made in 2005 [72]. The defective gene has been shown to be aristaless-like homeobox 4 (*ALX4*), a major regulator of hindlimb formation (J. Beever, personal communication). The deletion removes approximately one-third of the *ALX4* gene, including the upstream regulatory sequence, and involves approximately 46 kb. After identification of the initial deletion, it was noted that although the parentage of some TH calves was DNA-verified, some parents did not test positive for this deletion. Additional studies revealed that other TH carriers possess a larger deletion of 450 kb that overlaps the previous/smaller deletion, and removes four genes including *ALX4*. The later and larger identified deletion is rare and affected calves sired by carriers of that deletion are compound heterozygotes (heterozygous with the smaller mutation on one chromosome and the larger mutation on the other). Although "heterozygous," these calves are affected like those homozygous for the smaller deletion. The frequency of the larger mutation is so low, and the magnitude of the deletion so much larger, that it is unlikely that there have been any TH calves homozygous for the larger deletion. In Galloway cattle TH is most likely caused by a duplication of 20 bp in exon 2 of *ALX4*, resulting in a frameshift and substantially altered secondary and tertiary structure of the homeodomain, which supports the explanation for a loss of function [73].

Vertebral and Spinal Dysplasia

In 2010, early data emerged regarding newborn calves in Germany with short, crooked tails in the Holstein cattle breed [74]. Forty-one animals, all offspring of a single sire, showed pathognomonic tail malformations, providing highly significant evidence for a congenital inherited defect, which was subsequently termed vertebral and spinal dysplasia (VSD). The defect is characterized by vertebral (specifically tail) deformities and neurological dysfunctions with gait abnormalities of the hindlimbs. The deformities and neurological dysfunctions varied from very mild (only tail deformities) to severe (paraparesis). The malformations and dysfunctions associated with VSD, as well as the mode of inheritance and the genotyping of the suspected carrier sire, indicated that VSD is a defect previously not described in cattle. VSD is inherited in a dominant mode but shows incomplete penetrance of the phenotype. Genome-wide mapping, haplotype tracking within a large pedigree, resequencing of target genome regions, and bioinformatics analyses unambiguously confirmed the mutant c.196G allele as causal for this previously unknown defect. The non-synonymous VSD mutation is located within the highly conserved T box of the *T* gene, which plays a fundamental role in eukaryotic species body organization and vertebral development [74].

Summary

In recent years, several heritable defects have been identified that have had significant impact on specific cattle populations. Molecular geneticists, veterinary pathologists, breeders, and breed associations have cooperatively identified genetic disorders, characterized the pathology, determined the genetic mutations responsible, and developed tests to identify carriers. It is likely that the genetic mutations in other heritable congenital defects of cattle will be identified through similar collaborative efforts.

Although the investigation of heritable bovine fetal anomalies has often been left to those in academia (specifically, animal scientists and veterinary pathologists), many of the currently recognized inherited disorders of cattle would have gone undiscovered without the assistance of private practitioners and producers. For any surveillance program to be successful, recognition of a potentially heritable defect is the first step. The anomaly must be reported, appropriate samples collected and preserved, and pedigree information made available. Veterinarians in the field can play a pivotal role in discovery and surveillance by recognizing and reporting inherited defects of cattle.

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71

Abnormal Offspring Syndrome

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Introduction

Large offspring syndrome (LOS) refers to an overgrowth phenotype sometimes observed in cattle fetuses and newborns produced by the use of assisted reproductive technologies (ART) [1–4]. Enlarged body and limb size are the most commonly identified phenotypes [5]. However, LOS is not always characterized by overgrowth [3]; other phenotypes include abnormal organ growth, large tongue, muscle and skeleton malformations, umbilical hernias, abnormal placental vasculature, hydroallantois, and increased abortions throughout gestation [2–15]. These characteristics have prompted the use of a different designation for this syndrome, namely abnormal offspring syndrome (AOS) [3, 6, 14], a nomenclature that we will use throughout this chapter.

In this chapter we will discuss the current knowledge on ART-induced AOS. Even though AOS also manifests in cloned (somatic cell nuclear transfer [SCNT]) offspring [16, 17], and we present some data to that effect, most of the work presented here will focus on AOS resulting from common, non-invasive ART procedures. The reason for this is that AOS is an epigenetic syndrome (explained in detail in subsequent sections) and adding the confounding of somatic cell reprogramming (or lack thereof) complicates interpretation of results. When relevant, we will discuss findings from sheep as AOS has also been documented in this species, and from Beckwith–Wiedemann syndrome (BWS), a human congenital overgrowth condition which phenocopies AOS. In addition, we present evidence of spontaneous AOS in cattle [11] and provide two examples of financial loss incurred by the cattle industry as a result of this syndrome.

Assisted Reproductive Technologies (ART) in Cattle

In vitro production of embryos (IVP or ART) encompasses a series of laboratory procedures in which oocytes are collected from the ovaries either from live cows, often after superovulation [18], or from ovaries harvested at an abattoir [19]. If using live animals, superovulation may be followed by artificial insemination (AI) and embryo collection by uterine lavage with an embryo-compatible solution (i.e. flushing). Embryos may be transferred immediately (i.e. fresh) or frozen for future use. This procedure is referred to as multiple ovulation embryo transfer (MOET). Alternatively, oocytes may be harvested from ovaries *ex vivo* and cultured *in vitro* to promote their nuclear and cytoplasmic maturation [20, 21]. Then, the oocytes are incubated with spermatozoa, a procedure referred to as *in vitro* fertilization (IVF), and the putative zygotes are cultured in a medium designed to promote their development to the blastocyst stage. Variations on this basic procedure are many and depend on the laboratory, the technician, and the final purpose and have included the use of somatic cell coculture, serum supplementation, or a combination. Many media possess components that are undefined, such as serum or bovine serum albumin (BSA) (refer to section on "Serum Supplementation"). The use of defined media is favored by some [22], while others turn to the "physiological basics" by the use of reproductive fluids to supplement the culture medium in order to achieve an environment in which embryos are exposed to components found *in vivo* [23].

ART are commonly used in cattle for experimentation purposes [24] and are the method of choice when trying to improve genetic merit of the offspring in a shortened

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

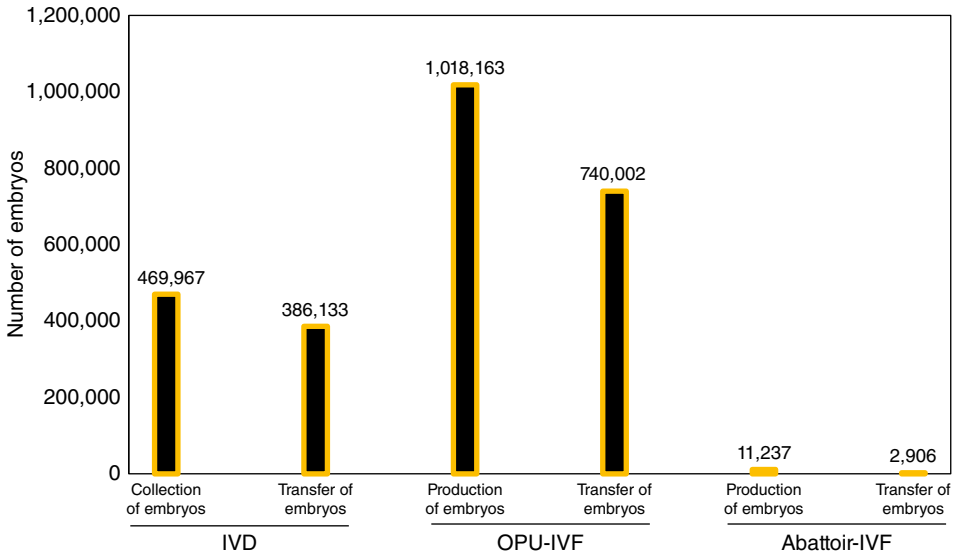


Figure 71.1 Number of bovine embryos produced and transferred worldwide in 2018. IVD, *in vivo* derived; OPU, oocyte pickup; IVF, *in vitro* fertilized (produced). *Source:* Data sourced from the International Embryo Technology Society 2018 Statistics of embryo production and transfer in domestic farm animals. https://www.iets.org/Portals/0/Documents/Public/News/IETS_Data_Retrieval_Report_2018.pdf?ver=e0U7aB054OEBv7BpZ_jufQ%3d%3d.

length of time when compared to natural reproduction [25]. Genetic merit is defined as the rank of an animal for its ability to produce superior offspring relative to other selection candidates [26]. In addition, ART can be used to produce genetically manipulated animals with improved production traits [27]. The first IVF-culture-transfer calf (bull calf born 9 June 1981) was reported by Brackett and colleagues in 1982 [28], and currently ~1.1 million transferable bovine embryos are produced each year (Figure 71.1).

Although widely used, these technologies are not without complications for the offspring. The observation that ART can induce the birth of abnormally large calves was first documented in the 1990s [9]. In that report [9] the authors noted: "At present, little information is available to identify the factors or possible mechanisms influencing excessive fetal growth." Even though this adverse outcome of IVP has been recognized and extensively studied since the early 1990s [2–4, 6, 13–15, 29–33], we are not yet able to predict which embryos are molecularly programmed to suffer AOS, as the etiology and the incidence of the syndrome are unknown. Increase in weight and size lead to dystocia in some instances and cesarean section is needed for delivery [34]. Of note is that overgrowth of the fetus is sometimes independent of gestation length [5]. A main hurdle to the understanding and perhaps elimination of AOS is that there is a lack of peer-reviewed data describing the status of AOS in the industry [7]. As a result, many believe this issue has been resolved, yet AOS continues to have detrimental effects on some ART-conceived offspring and its dam, which can result in financial loss to cattle producers and the embryo transfer industry (see examples in section "Economic Loss to the Industry as a Result of AOS").

Abnormal Offspring Syndrome – An Overgrowth Syndrome in Ruminants that Recapitulates the Human Syndrome Beckwith–Wiedemann (BWS)

Overgrowth syndromes refer to a group of conditions in humans with the common features of excessive growth which includes body weight, height, and organ size, and a higher risk of tumorigenesis [35]. One of the best characterized overgrowth syndromes, namely BWS (OMIM #130650), was first reported in the 1960s by Hans-Rudolf Wiedemann [36] and J. Bruce Beckwith [37], and was initially called EMG syndrome after its major symptoms (i.e. exomphalos, macroglossia, and gigantism) [38, 39]. The incidence of BWS is ~1 : 10 340 natural births [40]. Therefore it can be estimated that of the 3.7 million babies born that were conceived naturally in the USA in 2019 [41] ~357 were afflicted with BWS. This number may, however, still be an underestimation since individuals without obvious phenotypes may not ever be diagnosed. Common phenotypes observed in BWS are macroglossia (large tongue), macrosomia (large body size), lateralized overgrowth, ear malformations, abdominal wall defects (exomphalos/hernia), and hyperinsulinism [42, 43]. In addition, children with this

condition have an increased risk of embryonal tumors during early childhood [44]. Types of tumors observed include neuroblastoma, rhabdomyosarcoma, unilateral Wilms tumor, hepatoblastoma, adrenocortical carcinoma, and phaeochromocytoma, with Wilm's tumor of the kidney being a cardinal feature of this syndrome [43, 45]. Further, children conceived by ART have a higher likelihood of BWS than the naturally conceived population [46].

BWS is a heterogeneous molecular condition, as various epigenetic and genetic defects have been reported [43]. The first genetic linkage reported for BWS was with the insulin-like growth factor 2 (*IGF2*) gene [47], an imprinted gene found on chromosome 11p15 in humans. Subsequently, studies found genetic and/or epigenetic mutations on human chromosome 11p15 in BWS [48–50]. The syndrome is not limited to epigenetic alterations at 11p15 [51], and as such has been termed a multilocus imprinting disturbance. The main molecular defects of BWS can be categorized into three groups: (i) DNA methylation defects, (ii) changes of chromosomal contents, and (iii) gene mutations at chromosome 11p15. Among BWS cases with positive molecular diagnosis, the majority (65–73%) have DNA methylation defects, while the remaining cases may have some genetic abnormality (i.e. 16–33% have changes of chromosomal contents and 1–8% show gene mutations) which can ultimately affect imprinted gene expression (i.e. resulting in a secondary epimutation [52]) [53–63].

Shared phenotypes observed between AOS and BWS are large tongues, large body size, abnormal organ growth, abdominal wall defects, hypoglycemia, and abnormal placental development [3, 15, 43]. As the causes of BWS at the molecular level have been largely attributed to epigenetic defects, which include alterations of DNA methylation and loss-of-imprinting (LOI) [43], we will discuss these epigenetic modifications/mechanisms in the next section.

Epigenetics, DNA Methylation, and Genomic Imprinting

Epigenetics is the study of chromatin behavior and all the modifications, mechanisms, and molecules that orchestrate gene expression, gene silencing, and genome integrity. DNA methylation refers to the addition of a methyl group (CH_3) to the fifth carbon of cytosines (in most cases at CpG dinucleotides) by the DNA methyltransferases (DNMTs). The "p" stands for phosphodiester bond and this nomenclature is used to indicate that a guanine follows a cytosine in the DNA sequence. This epigenetic modification is inherited through cell division and is critical for embryonic and fetal development and reprogramming of the germ cell lineage [64]. In addition, DNA methylation has roles in gene expression by regulating binding accessibility of proteins to DNA [65], is involved in the control of genomic imprinting [66], retrotransposons repression [67], and dosage compensation (i.e. X-chromosome inactivation) [68]. Further, an inverse correlation exists between DNA methylation at the promoter, transcription start site, and first exons of genes, and transcript levels [69]. Methylated cytosines promote a silent state of genes by attracting methyl-CpG-binding domain protein which interferes with the binding of transcriptional regulators and forms complexes with enzymes which can promote a silent state of chromatin [70, 71]. Other epigenetic modifications/factors that are involved in the regulation of chromatin, although not discussed in detail here, include histone post-translational modifications [72], histone variants [73], long and short non-coding (nc) RNAs [74, 75], and the epigenetic writers, readers, and erasers of histone proteins and DNA [76]. The epigenome is responsive to endogenous and exogenous environments and, as such, is labile [77]. Of relevance to this review is that artificial environments associated with ART have been shown to have profound effects on the epigenome of gametes and embryos [78–80].

Genomic imprinting is a series of precisely regulated epigenetic processes that lead to parental-allele-specific expression (Figure 71.2) of a subset of genes in mammals [81–83]. Since the discovery of the first three imprinted genes in mouse, namely, *Igf2*, *Igf2r*, and *H19* in 1991 [84–86], more than 100 genes (the number will depend on the species) have been identified to be imprinted in mammals [87, 88]. Most of the identified imprinted genes are located in clusters containing two or more genes subject to genomic imprinting. Within each cluster, a discrete region of DNA, termed as the imprinting control region (ICR), regulates the proper allele-specific expression of imprinted

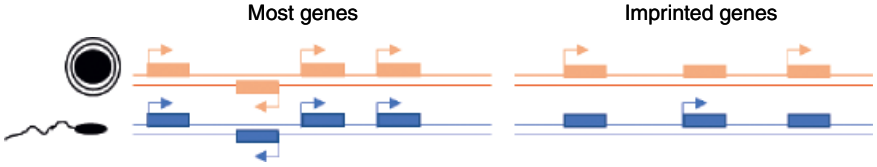


Figure 71.2 Gene expression in mammals. Shown is a pictorial representation of the expression of most genes which are expressed in a biallelic manner and imprinted genes which are expressed in an allele-specific (monoallelic) non-random manner.

genes. ICRs are marked with parental-allele-specific DNA methylation, which distinguishes the parental alleles in the same nucleus. The allele-specific DNA methylation at ICRs is established in the germline in a sex-specific manner [89]. In mice, male germline methylated ICRs are established before birth, while acquisition of the maternal-specific DNA methylation occurs postnatally during oocyte growth. These gametic DNA methylation imprints escape the wave of global DNA demethylation that occurs after fertilization and are maintained in somatic cells throughout development. Further, as part of the global DNA demethylation that occurs in primordial germ cells, methylation imprints are erased in order to get reestablished in a sex-specific manner [90].

The importance of genomic imprinting in mammalian development was first demonstrated by pronuclear transplantation experiments, in which androgenetic and gynogenetic mouse embryos resulted in embryonic lethality [91, 92]. Conceptuses derived from zygotes which contained two maternal genomes were smaller than the biparental fetuses and had an undeveloped placenta, while those containing two paternal genomes had a severely retarded fetus but a well-developed placenta [91, 92]. Therefore genomic imprinting may be regarded as the block to parthenogenesis in mammals [93]. Investigations of imprinted gene function also demonstrated the significance of genomic imprinting in mammalian development. For example, mice with maternal transmission of the non-functional allele of *Igf2*, a paternally expressed fetal growth factor, showed normal growth, but paternal transmission of this mutant allele had deficient growth that was similar to *Igf2* null mice [85]. Although less effort has been put forth in determining the function of imprinted gene products than in understanding the mechanism of imprinting, these genes emerge as important players in numerous biological processes including fetal growth, placental development, neural development, metabolism, and behavior [94, 95].

Abnormal Offspring Syndrome

The first cases of fetal overgrowth and developmental abnormalities in cattle were reported by Willadsen et al. [17]. In that study, 8–64 cell-stage embryos from beef and dairy breeds were collected by flushing superovulated cattle and embryos split into individual blastomeres for cloning experiments. Interestingly, those authors report in their discussion that when the manuscript was being written (i.e. 1 October 1990) some calves from the study had already calved themselves, a point that indicates that these studies were performed in the mid-1980s and that AOS was already being observed at that time – information we have also learned through personal communications from individuals working with SCNT at the time. In the Willadsen study, 8/11 cases of abnormal offspring observed had twins that were of normal appearance. This is reminiscent of the situation in humans in which monozygotic twins can be discordant for BWS [96], pointing at the epigenetic nature of the syndrome. Overall, AOS is associated with decreased pregnancy rates, higher loss of embryos, increase in fetal or neonatal weights, increased organ size, abnormal limb and spinal cord formation, increased incidence of hydroallantois, prolonged gestation, and ultimately poor outcome of the offspring, in part due to dystocia [2, 6, 9, 17, 97–99].

Treatments Associated with AOS

Serum Supplementation

For many years, the field has recognized that augmentation of embryo culture media with serum may induce an overgrowth phenotype (Table 71.1) [31, 33, 110]. For example, in one study, 25% of ovine embryos cultured *in vitro* with serum supplementation developed as large offspring [32]. In addition, the presence of serum in the culture medium has been associated with increased body weight and gestation length in sheep, which, similar to cattle, suffer ART-induced AOS [97]. Serum can also result in increased organ size (a phenotype of AOS [31]).

Supplementation with serum during *in vitro* embryo culture has been historically used to stimulate blastocyst formation (see Figure 71.3 for an example from our laboratory) [123, 124]. However, while supplementing the culture medium with fetal calf serum and BSA was found to improve and accelerate blastocyst yield by day 6, serum can also decrease embryo survival rate [100, 101]. Different culture conditions and supplementation with serum have been reported to cause transcript abundance changes of several developmentally important genes involved in cell–cell junctions, transport, RNA processing, and stress in bovine embryos [102, 103]. In addition, alterations of imprinted- and non-imprinted gene expression as a result of *in vitro* embryo production have been reported in numerous studies [88, 102–108, 111, 112, 125–128]. For example, a twofold increase of *IGF2* (a paternally expressed fetal growth factor) transcript was detected in liver of day 70 bovine fetuses that were cultured in medium containing estrus cow serum when compared with the serum-restricted group [29]. These fetuses presented loss of methylation (LOM) at the insulin-like growth factor 2 receptor (*IGF2R*) ICR, which was correlated with reduction of the *IGF2R* transcript and protein levels. IGF2R is a maternally expressed [15, 109] scavenger receptor of the paternally expressed fetal growth factor IGF2.

Table 71.1 Examples of publications in which AOS is seen.

Species	Environment	Reference number	First author	Publication date
Cow	Serum	[100]	Carolan et al.	1995
Cow	Serum	[101]	Thompson et al.	1998
Cow	Serum	[102]	Wrenzycki et al.	1999
Cow	Serum	[29]	Blondin et al.	2000
Cow	Serum	[103]	Wrenzycki et al.	2001
Cow	Serum	[104]	Rizos et al.	2003
Cow	Serum	[105]	Rizos et al.	2004
Cow	Serum	[106]	Corcoran et al.	2006
Cow	Serum	[107]	Smith et al.	2009
Cow	Serum	[108]	Driver et al.	2012
Cow	Serum	[15]	Chen et al.	2013
Cow	Serum	[109]	Bebbere et al.	2013
Cow	Serum	[110]	Cagnone et al.	2014
Cow	Serum	[88]	Chen et al.	2016
Cow	Coculture and serum	[111]	Chen et al.	2017
Cow	Coculture and serum	[2]	Farin et al.	1995
Cow	Coculture and serum	[99]	Schmidt et al.	1996
Cow	Coculture and serum	[112]	Rizos et al.	2002
Sheep	Coculture and/or serum	[113]	Cordova et al.	2014
Sheep	Serum	[97]	Thompson et al.	1995
Sheep	Serum	[33]	Rooke et al.	2007
Sheep	Coculture	[30]	Maxfield et al.	1997
Sheep	Coculture	[114]	Maxfield et al.	1998
Sheep	Coculture or serum	[115]	Holm et al.	1996
Sheep	Coculture and serum	[116]	Gondolfi et al.	1987
Sheep	Coculture or serum	[31]	Sinclair et al.	1999
Sheep	Coculture and/or serum	[32]	Young et al.	2001
Sheep	Maternal diet, serum, coculture	[117]	McEvoy et al.	1997
Sheep	Asynchrony	[118]	Wilmut et al.	1981
Sheep	Asynchrony	[119]	Kleemann et al.	1984
Sheep	Asynchrony	[120]	Maxfield et al.	1998
Sheep	Asynchrony	[121]	Sinclair et al.	1998

AOS has been observed in offspring that were not exposed to serum supplementation during embryo culture; however, serum is still often used to supplement the maturation medium in some of those cases (personal communication). A question that remains unanswered is why/how is serum promoting AOS. This is a very complicated question to address as the exact nature of the molecule(s) that can affect the epigenome and/or gene expression is not known and could be diverse. For example, studies in which the composition of bovine serum was determined showed that it is composed of sundry hormones, vitamins, growth factors, lipids, carbohydrates, and transport proteins, among other unidentified biomolecules [129–131]. In addition, these components may change between batches as shown for fetal calf serum [131], a fact that could be attributed to differences in maternal diet, breed, gestational age, and seasonal environmental factors, among others.

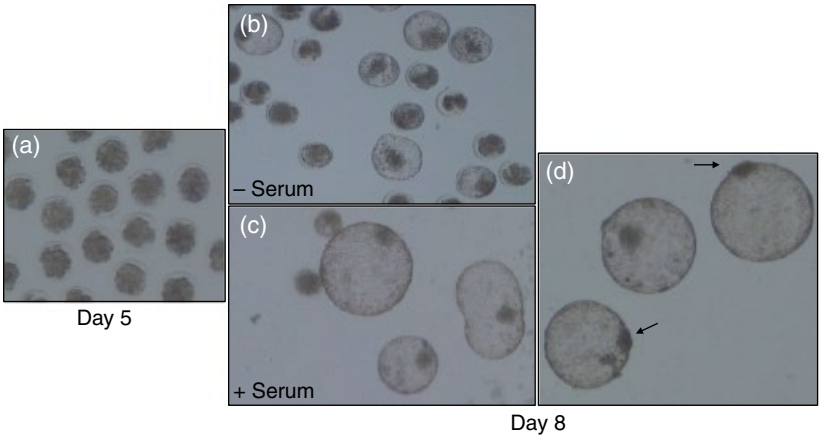


Figure 71.3 Why add serum to the culture medium? Shown are day 5 (left; a) and day 8 (right; b, c) bovine blastocysts which were processed together (*in vitro* maturation, IVF, and embryo culture) by us until day 5 (a), at which time they were separated into two groups. One group received 10% estrus cow serum (c) whereas the other group was not supplemented (b). Embryos in the serum group were more advanced in stage (note that they have hatched from the zona pellucida) and there were more at the blastocyst stage when compared to the control group. In addition, some of the hatched blastocysts in the serum group had a protruded embryonic mass (arrows; d). Pictures are to scale except (d) which is magnified to show protruding embryonic mass. *Source:* Pictures are courtesy of Rocío Rivera.

Coculture with Somatic Cells

Coculture of embryos with various types of cells has also been used to increase blastocyst yield (Table 71.1) [113, 116, 122]. Similar to what has been found with serum supplementation, overgrowth [30, 115] and increased gestation length [115] have been reported for ovine embryos that were cocultured with granulosa or oviduct epithelial cells. In addition, the size of the primary muscle fibers (which form during the first wave of myogenesis) and the ratio of secondary to primary fibers of the cocultured fetuses were greater than the controls, which indicates that hypertrophy of the primary fibers and hyperplasia of the secondary fibers are associated with the increased body weight observed in these fetuses [114]. A confounding element of some of the studies in which AOS was observed is that the culture medium was also supplemented with serum [2, 99].

Maternal Diet

Another factor associated with AOS is maternal diet (Table 71.1). For example, substantial increase in embryo mortality and one case of fetal overgrowth were found in ewes fed excess amounts of urea (excess rumen-degradable nitrogen) from 21 days before mating to day 63 of gestation when compared to controls [117]. The average weight at birth for controls was 7.16 kg and the weight of the single lamb (male) of the high urea group was 10.1 kg. The cause behind the overgrowth is not clear but may be due to the higher systemic urea concentration, including the uterooviductal microenvironment [117], and/or postprandial daily elevations in plasma ammonia observed in the ewes. In addition, in that study, embryos collected on day four and cultured to the late blastocyst stage were observed to be more developmentally advanced and have increased metabolism than their control counterparts. Of note is that in the study, embryos were cocultured with oviductal cells in the presence of 10% fetal serum. Nonetheless, these authors still observed an increased rate of metabolism and development of preimplantation embryos, suggesting that ammonia buildup in the culture medium may be in part responsible for the observation, and noted that in their previous study [132] they observed that serum supplementation of medium increased the levels of ammonia *in vitro,* similar to the levels found in the urea-fed ewes. They go further and propose ammonia as a causal agent for the result on embryonic development, which may be compared to the situation in mouse in which high levels of ammonium during embryo culture resulted in fetal abnormalities [133].

Asynchrony of Maternal Environment

Asynchrony between embryo age and maternal environment has also been reported in sheep to result in larger than normal offspring [118] or to have altered myogenesis (Table 71.1) [114]. In one study, day 3 embryos were placed in a day-6 uterus for a period of three days and then transferred into another recipient synchronous with the embryo (i.e. day-6 uterus and day-6 embryo) [118]. In the asynchronous fetuses, abnormal increase in the weight of the offspring was observed when compared to the completely synchronous double embryo transfer group. The cause for this observation may be that an advanced uterine

environment provided one or more secretory signals or hormones earlier than required by the embryo. To this effect, increased fetal growth was observed in pregnant sheep primed with progesterone (controlled internal drug release [CIDR]) for three days after ovulation when compared to control [119]. The lambs were heavier (weight in grams = 220.8 ± 3.2 and 247.6 ± 4.1 for control and progesterone group, respectively) and longer (crown–rump length in millimeters = 204.0 ± 1.1 and 212.1 ± 1.4, respectively) at birth, which they proposed may be due to the advanced maternal environment experienced by the embryos during preimplantation development. In another study the authors found that while muscle weight was not altered in the asynchronous group when compared to the control, the ratio of secondary to primary fibers in sections of plantaris muscle was increased, as well as the number of muscle fibers, a phenomenon they hypothesized may involve hyperplastic growth in the secondary fibers [120]. The latter hypothesis is interesting as the study by Wilmut and Sales [118] showed that in the asynchronous transfer group, embryos were larger, more had hatched from the zona pellucida, and one had a clear embryonic disk reminiscent of a day-10 sheep embryo, also suggesting hyperplasia as a result of factors present in the advanced uterine environment. We also observed that the embryonic mass was protruding from some of the hatched blastocysts cultured in serum (Figure 71.3d). It should be noted, however, that increased fetal weight has not always been observed in studies of similar design [121].

Molecular Alterations Associated with AOS

It has been more than 20 years since the hypothesis that epigenetic factors play a role in AOS was first pointed out [5] and almost 30 years since the first indication of AOS being an epigenetic syndrome was shown [17]. In the past two decades, many researchers have demonstrated correlations between abnormal phenotypes and epigenetic aberration in AOS [32, 134–141]. The onset of these aberrations has been proposed to be the results of incorrect reprogramming of epigenome in early embryos during critical stages of the preimplantation period [3]. Genome-wide reprogramming of the epigenome during early embryo development is a conserved mechanism in ruminants [142, 143]. The reprogramming will not affect some genomic sequences such as imprinted methylation and histone modifications retained in the paternal genome which are inherited by the zygotes [144–146]. Changes in environmental factors, including chemicals, oxygen level, and temperature, may affect the success of the reprogramming procedure. Studies have shown that level of non-esterified fatty acids in the culture medium affects global DNA methylation in embryos [147]. Supplementing vitamin C in the culture medium has been shown to improve the reprogramming of DNA methylation at loci initially being aberrant in SCNT-derived embryos [148]. Level of oxygen exposure affects the expression of the demethylase *TET* family, de novo methyltransferase *DNMT3A*, and its cofactor *HELLS* in bovine embryos, which may further alter the DNA methylation [149]. Studies have also shown that active DNA demethylation followed by de novo methylation in paternal pronuclei of zygotes can be perturbed by heat-stress spermatozoa [150]. Since DNMTs are directly responsible for de novo methylation and maintenance, it is highly possible that their expression and function are disrupted in AOS. One study on SCNT-derived fetuses showed alteration of DNA methylation at the potential promoter regions of *DNMT3a* and *DNMT3b* [151].

DNA methylation affects the accessibility of DNA and therefore can regulate gene expression. Like humans, the global DNA methylation pattern in bovines is high in gene body and low in promoter regions [111, 134]. Although this pattern remains the same, the level of DNA methylation across the gene body and intergenic regions can be different in AOS. A metagene analysis revealed that DNA methylation level of gene region (defined as gene body ± 2 kb) in cotyledon of deceased SCNT-derived calves is lower than naturally conceived calves [134]. Another study of IVP derived bovine AOS fetuses demonstrated global aberration of the DNA methylome in these fetuses, and the majority of changes occur within gene body [111]. However, only a small percentage of differentially expressed genes can be directly correlated to adjacent (<20 kb) DNA methylation changes, indicating the involvement of other mechanisms [111]. Other groups have reported large changes in intragenic DNA methylation level [140]. The impact of *in vitro* culture of the embryo on the methylome has been shown in a stage-specific manner [152]. Higher ratio of hypomethylated versus hypermethylated regions within intronic, exonic, promoter, and proximal promoter regions was found in embryos transferred as zygotes, the reverse was found in embryos transferred at 4-cell and 16-cell stage, and about equal amount of hypermethylated and hypomethylated regions were found for embryos transferred as blastocysts [152].

Histone modifications, including methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation, play important roles in replication, transcription, heterochromatin formation, chromatin compaction, and DNA damage repair [153]. Studies have shown genome-wide elevation of histone H3 lysine 9 dimethylation and acetylation in SCNT derived embryos [154]. Increased histone H4 acetylation has been reported for imprinted genes, including *IGF2* in deceased SCNT derived fetuses [135].

Increased permissive mark histone H3 lysine 4 dimethylation and decreased repressive mark histone H3 lysine 9 dimethylation were associated with increased expression of *PHLDA2* in placenta of SCNT derived fetuses [139]. It is important to note that some studies have also shown that during SCNT some original somatic histone modifications can escape reprogramming and be inherited by the embryos [141]. This aberration of histone modification can further affect gene expression and genomic imprinting [141].

Non-coding RNAs, especially long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), also play important roles in epigenetics and have been shown to be involved in AOS. lncRNAs have function in regulation of gene transcription, working as a precursor or sponge for small RNAs and remodeling broad chromatin domains [155]. Misregulation of lncRNAs in AOS, for example *KCNQ1OT1*, has been reported in many studies [15, 138, 156]. In addition, alteration in expression level of hundreds of miRNAs has been shown in mid-gestation IVP derived bovine AOS fetuses [136]. These affected miRNAs show clustering tendency in the genome, especially from the DLK1-DIO3 genomic imprinted cluster, also shared by BWS [136], and some are associated with adjacent DNA methylation changes. In consideration of miRNAs' most important function, namely inhibiting translation, these misregulated miRNAs could lead to expression changes of hundreds to thousands of genes at the protein level while having no or little effects on mRNA level.

Genomic imprinting regulates parental origin-specific expression of imprinted genes. We have reported that AOS is a global LOI condition [138]. Disruption of normal DNA methylation pattern at ICRs of imprinted genes and the corresponding LOI and change in expression level have been widely reported in overgrowth syndrome in bovines and humans. For example, there is a gain of methylation at H19/IGF2 ICR in BWS, as well as *H19* downregulation and *IGF2* overexpression [29, 111, 134, 137, 157–159], LOM at KvDMR1 and *CDKN1C* downregulation [15, 156], XIST DMR LOM and *XIST* upregulation [137, 157, 160], IGF2R ICR LOM and *IGF2R* upregulation [32, 137, 138, 157, 158, 161, 162], and misregulation or LOI of other imprinted genes [138, 147, 159, 163–165]. These imprinted genes have critical roles during early embryo development, and the effect of their dysfunction will be aggravated by downstream signaling pathways and can finally result in global gene misregulation. Conversely, upstream misregulated genes and epigenetic signatures can result in LOI. When LOM at DMRs occurs in early embryonic stages due to *in vitro* culture or SCNT, the methylation may be regained in a later stage partially or completely, suggesting the existence of repairing mechanisms [159], although ICR methylation is only deposited during gametogenesis and once lost cannot be regained.

IGF2 is a paternally expressed gene and functions in promotion of growth and development of the conceptus [166]. Similar to human and rodent models, the mRNA of *IGF2* can be transcribed from multiple promoters in a tissue- and developmental stage-specific way in bovines [167–169]. The dramatic influence of *Igf2* on body weight has been widely studied through null mutation or overexpression experiments in rodents [170–172]. *IGF2R* is maternally expressed and its protein binds to IGF2 with high affinity and results in degradation of excess IGF2 [173]. In addition to body weight changes, *Igf2r* null mutation or overexpression studies in rodents have shown similar abnormal phenotypes observed in AOS, including omphalocele, visceromegaly, and placentomegaly [174, 175]. *CDKN1C* is maternally expressed and encodes an inhibitor of cyclin-dependent kinase. Knockdown of *CDKN1C* in bovine zygotes significantly reduced the development rate to blastocysts [176]. *Cdkn1c* is required for normal embryo development and rodents with null mutations on this gene exhibit similar abnormal phenotypes found in AOS, including umbilical hernia, omphalocele, macroglossia, and placentomegaly [177–179].

As mentioned, very few correlations were found between altered gene expression and DNA methylation status at promoter/body of the gene regions (i.e. short range interactions) in these fetuses [111, 136]. This is not surprising, however, as several reports show that DNA methylation at remote regulatory regions (i.e. enhancers) is more explanatory of altered gene expression [180, 181]. For example, methylation of DNA can cause changes in binding efficiency of transcription factors (activators and repressors) and architectural proteins to DNA, which affect enhancer/silencer activity and chromosomal conformation, respectively [180, 181]. Together, these alterations can affect gene expression at megabase or interchromosomal levels [180–183] – a question we are currently addressing in the laboratory.

Effects of *In vitro* Maturation of Bovine Oocytes: Association to the Epigenome

A question that remains is "how early are gametes and/or embryos programmed to suffer AOS?" A study performed to analyze the effect of *in vivo* versus two different *in vitro* maturation media showed that *in vitro* matured oocytes had significant upregulation of *H19*, *IGF2R*, and *PEG3* mRNA levels [184], genes that are known to be paternally *(H19, IGF2R)* and maternally *(PEG3)* imprinted. Moreover, *DNMT1a*, *DNMT3a*, and *DNMT3b* mRNA were upregulated in both *in vitro* maturation groups, suggesting that

DNA methylation factors (proteins) could have been altered. However, the researchers did not find any differences in methylation levels of aforementioned genes in the maturation treatments [184]. The fact is that each maturation medium will have its specific effects on gene expression in the oocyte. For example, maturation of oocytes in medium containing non-esterified fatty acids had altered transcript levels of *DNMT3a* and *IGF2R* [185]. This may be associated with a global effect on the epigenome [186] which results in global misregulation of transcripts, including those involved in gene expression, transcription initiation, and energy metabolism [187]. As noted, the epigenome is responsive to environment, including internal physiologic state and external stimuli [186–188], and as such can be altered. To that effect, *in vitro* matured bovine oocytes showed loss of immunoreactive acetylation of lysine 12 in histone 4 (H4K12ac) following germinal vesicle breakdown, suggesting an activation of gene expression for the oocyte to resume to meiosis [186]. In addition, a DNA methylome study demonstrated differential methylation between those oocytes that were matured *in vitro* when compared to *in vivo* controls [189]. Altogether, these findings suggest that *in vitro* maturation of bovine oocytes does not completely mimic the *in vivo* model and may induce aberrations in DNA methylation and/or gene expression that could affect long-term embryonic or fetal development, which ultimately could lead to AOS.

Spontaneous AOS

Congenital abnormality has been observed from time to time in the field, either presenting as abortion or present at term birth. In addition, perinatal mortality, death occurring prior to, during, or within 48 hours of calving, is a recognized problem in the cattle industry [190]. In developed countries, 25–46% of perinatal deaths in cattle are the result of dystocia, and in the USA, 32% of perinatal deaths are due to unknown causes [190]. Dystocia is defined as an abnormal or difficult birth at any stage of labor. Dystocia has a direct negative impact on calves [190, 191], dam survival, reproduction performance [192], and milk production [193]. Replacement of stillborn calves is a substantial cost to the cattle industry and can amount to more than USD $125.3 million per year in the dairy industry [194]. One of the main causes of dystocia is fetal–maternal size mismatch [195], such as with abnormally large fetuses.

AOS manifest as large for gestational age calves have long been recognized [196]. With the advent of ART, AOS became almost exclusively associated with calves derived from these techniques [4]. However, it appears that AOS occurs spontaneously in non-ART calves as well, a phenomenon that we have coined as spontaneous LOS (SLOS) (Figure 71.4) [11]. Differentiating between AOS and other etiologies of fetal oversize can be challenging. When determining the cause of these conditions it is best to categorize them by gestational length – prolonged versus appropriate.

There is no established definition for post-term or prolonged gestation in cattle. In humans, births more than two weeks over the expected delivery date are considered post term and are independently associated with reduced neonatal outcomes [197]. Most cattle do not exceed a gestation of 290 days, and therefore a gestation length exceeding 300 days can be considered prolonged [198]. Gestation length, even when in the normal range, directly correlates to birth weight (i.e. longer gestation results in greater birth

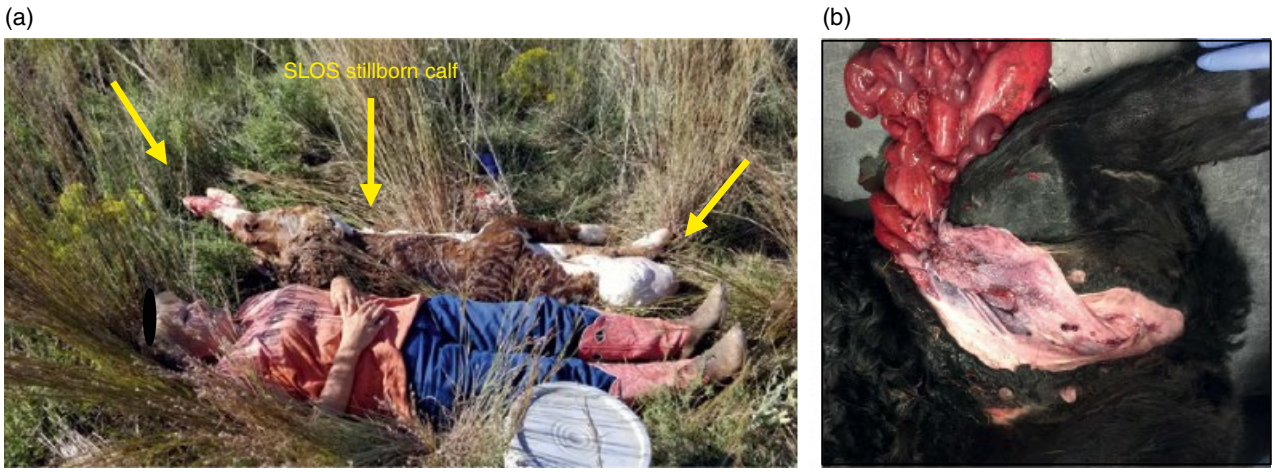


Figure 71.4 Examples of spontaneous AOS. (a) Dead SLOS calf at birth (yellow arrows are pointing to the calf). Next to the calf, a woman lying down for size comparison. (b) A newborn Angus SLOS calf. The calf was euthanized at birth and diagnosed with cloacal exstrophy, omphalocele (exomphalos), and diastasis recti (abdominal separation). *Source:* Pictures are courtesy of Rocío Rivera.

weight) [199]. As such, calves from prolonged gestation are almost invariably large (rare exceptions reported), and in general have poor survival outcomes. These calves may also have long hair-coats, overerupted teeth, and overgrown hoof capsules and have been reported to weigh from 59 to 98 kg. Once identified, these calves require delivery by cesarean section in order to salvage the dam.

Prolonged gestation and failure to initiate parturition have been documented in Ayrshire, Holstein-Friesian, Guernsey, Jersey, Swedish Red and White, and Belgian Blue breeds [200–204]. Prolonged gestation in these cases is secondary to adenohypophyseal hypoplasia/aplasia, cerebellar hypoplasia, and adrenal hypoplasia of the fetus, collectively referred to as "pituitary giants." Dysfunctional fetal hypothalamic–pituitary–adrenal (HPA) axes results in a failure to initiate parturition, and consequently fetal oversize. A hereditary component has been suspected in many of these cases; however, putative genetic variants have not yet been identified. Infectious and toxic agents such as Akabane virus, bluetongue virus, bovine viral diarrhea virus, and *Veratum californicum* may also prolong gestation through impairment of the fetal HPA axis and should be considered in these cases [205].

Fetal giants have been rarely recognized in calves that are born at normal gestation length. No specific descriptions of these calves have been made, with calves over 59 kg (130 lb) at birth considered to be fetal giants [196]. Using this weight as a threshold is likely to remove the effect of breed on inclusion as a fetal giant, as this weight is very large regardless of breed. In humans a neonate is considered large for gestational age if their weight exceeds the 97th percentile for a given gestational age (approximately 10 lb at term), regardless of race [206]. What is considered large for gestational age and a fetal giant in cattle is still yet to be fully agreed; however, we have defined it as a calf that is 75% bigger than expected (approximately 55 kg for Angus and 68 kg for Holstein).

SLOS calves have been observed in both Holstein and Brown Swiss, with calves of both male and female sex. These calves were all extremely large (63–86 kg) and required delivery by cesarean section. Calves affected by this condition may have a variety of other clinical features including macroglosia (enlarged tongue), ompaholocele (umbilical hernia), linear folding of the ears, and flexural and angular limb deformities. The survival of these calves is poor, with the majority dying within the first few days of life. We analyzed the muscle of one of these SLOS for LOM at KvDMR1 and observed that as in ART, SLOS appears to have an epigenetic association (Figure 71.5).

There are currently no estimates for the number of SLOS calves born each year. If we assume that ART-associated AOS occurs at a similar rate to non-ART-related BWS in humans (1/10 340 live births [207]), it is estimated that approximately 5000–10 000 calves are born each year in the USA with SLOS [208]. While the absolute number is unknown, the potential economic impact of these calves is

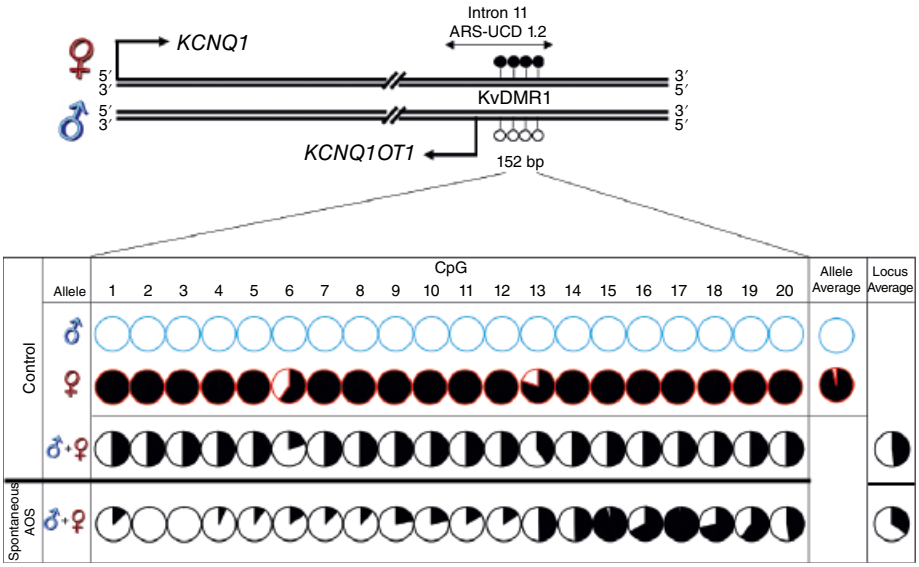


Figure 71.5 Loss of methylation at KvDMR1 in SLOS. Each circle represents a CpG dinucleotide in 5′ to 3′ direction with the 152-bp amplicon. Open circles = unmethylated CpG; filled circles = methylated CpGs. NCBI Reference Sequence: NC_037356.1. ARS-UCD1.2 Chromosome -29: 48908288–48908439. Primers used to amplify the bisulfite converted DNA were: forward: GTG AGG AGT ATG GTA TTG AGG, and reverse: TCA ACC CTC TCA ACC AAT AAC. Product size = 385 bp (only 152 bp are shown). Annealing temperature = 59 °C and number of CpG = 38. The sample used for control is the tongue of fetus C010 ([15]) and the muscle is of a SLOS (53-kg Holstein calf which was collected at birth and deemed "abnormally big" by the veterinarians at UC Davis).

likely to be significant. As these calves invariably result in dystocia requiring resolution by cesarean section, it is estimated that AOS costs cattle producers $3–5 million per year [209]. This does not, however, account for more mildly affected individuals. Milder case presentation has hampered the estimation of incidence of similar conditions in humans, as they are rarely investigated, and therefore the true incidence is likely to be much higher [210]. Given the potential for mildly affected individuals to exist in bovine populations, this condition may be more pervasive. There is strong evidence that calf mortality is increasing and we propose that this may be in part due to mild SLOS. Calf birth weight has a large bearing on neonatal calf outcomes, with increasing birth weight positively associated with increased risk of calf mortality [211]. This has an economic impact, but also a welfare implication for affected calves. These calves are not only at greater risk of mortality, but also will be more likely to have failure of passive transfer of immunity, require more medical interventions, and have indicators of physiologic stress [212]. Large for gestational age calves are likely to be encountered by most bovine practitioners. SLOS should be considered when evaluating these cases and when counseling clients.

Economic Loss to the Industry as a Result of AOS

There are no published data on the economic loss experienced by the industry as a result of AOS. A main phenotype of the AOS is increased bodyweight and size of the calves which can lead to dystocia requiring surgical fetal extraction [34]. LOS calves can also have an enlarged tongue at birth, which can lead to difficulty breathing and suckling, resulting in lower neonatal viability. Furthermore, the cost of veterinary services can bring economic burden to the farmer. Given the lack of information on economic loss to producers, we asked an embryo transfer technician and a farmer who each experienced AOS to provide actual and projected economic loss as a result of SLOS. Below we provide the information given and summarize their losses in Figure 71.6.

Example 1 (Oklahoma, 2019)

> Six continental breed composite embryos. Conventionally derived embryos from the same flush that were transferred fresh not frozen. First recipient goes into partial paralysis due to oversized calf's head pinching nerve in birth canal. I induced the calf 16 days prior to due date. This first calf weighed 124 lb at time of calving and did not survive. With an estimated growth of 1.5 lb/day, conservatively, in the last month of gestation it can be assumed the calf would have weighed roughly 148 lb. This encouraged me to palpate the remaining five recipients to estimate size of fetus. Two days after the first, I induced the remainder of the recipients. At 14 days premature I had 5 calves all requiring either an episiotomy or cesarean to deliver. Of these 5, only 2 calves survived. The 2 surviving

Example 1. Continental breed composite

Total loss	$28,832.37
Cow related loss	$5000 (4 x $1250 to replace with open 6 y/o cows)
Calf related loss	$7200 (4 x $1800 for embryo calves)
Medical related loss	$1782.37
Future loss	$14,850 (4 cows x 4 more calves per cow x 450 lbs x $1.65)





Example 2. Charolais

Total Loss	$29,200
Cow related loss (Current + Predicted)	$14,500 (dam valued at $2600, sold for salvage $500 + value of next four calves as yearlings were expected –$12,400)
Calf related loss (170 lbs = 77 kg)	$2,600 (Average selling price of their yearling bulls)
Sire related loss	$11,000 (sire cost – had to sell to market after SLOS son)
Medical related loss	$1,100



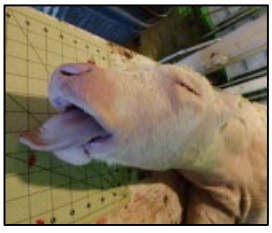


Figure 71.6 Examples of calf death, adverse outcomes to the dams, and financial loss to producers as a result of LOS.
Source: Pictures are courtesy of Rocío Rivera.

calves consisted of a 113-lb heifer and a 122-lb bull. The 3 deceased calves consisted of a 118-lb heifer, a 127-lb bull, and one bull that weighed 142 lb that lived for roughly 24 hours before passing. Four of the recipients also perished in the event.

Example 2 (Missouri, 2019)

Probably the largest financial impact was to the herd reputation. I gave $11 000 for the herd sire and since the birth of this calf have had to send him to market, no one wants to buy a bull from a sire that sired a 171-lb calf. Also, 60% of my female herd are daughters of this sire – is the worth of these animals now in question? I have noticed less local buyer traffic for my purebred seed stock cattle since the word of our farm having a 171-lb dead calf. This total impact is very high but difficult to place a figure on at this time.

Summary

The observation that ART can induce the birth of abnormally large calves was first documented in the 1990s [9]. However, we are not yet able to predict which embryos are molecularly programmed to suffer LOS as the etiology and the incidence of the syndrome are unknown. A main hurdle to the understanding and perhaps elimination of LOS is that *there is a lack of peer-reviewed published data describing the current status of LOS problems in the commercial embroy transfer–IVP industry* [7]. Overgrowth syndrome occurs naturally in ruminants and humans [11, 40] and both have been shown to increase in incidence in offspring conceived by ART [46, 132]. This fact speaks to the epigenetic nature of this condition. AOS can cause financial burden to genetic improvement and embryo transfer companies as well as cattle producers. Often both, the dam and the calf die as a result of dystocia. Early identification of AOS would allow early elective termination of the pregnancy in order to prevent the aforementioned monetary losses and animal welfare concerns associated with AOS.

Acknowledgments

RMR is supported by the Agriculture and Food Research Initiative competitive grant no. 2018-67015-27598 from the United States Department of Agriculture National Institute of Food and Agriculture. EJSM is supported by NIH NIGMS R25GM056901. We are grateful and indebted to those people who provided pictures and financial loss summaries for this chapter; their information is not provided to protect their anonymity. ORCID: RMR – 0000-0002-9832-9618, CGD – 0000-0003-1503-1419, BP – 0000-0001-8346-5337, YL – 0000-0003-3062-4626, EJSM – 0000-0002-1925-2940.

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Section III

The Neonate

72

Management to Decrease Neonatal Calf Loss in Beef Herds

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Introduction

Calving is a highly anticipated event for the beef cow-calf producer. The first few weeks of each calf's life are critical to its future health and productivity, and collectively the health and productivity of the herd, as well as the financial success of the enterprise. Successful calving seasons are planned in advance, with much consideration to minimizing the likelihood and costs of the known hazards of this phase of cattle production. Important hazards during the calving season are birthing problems, dangers from the environment, and sickness or death from disease. Understanding these hazards allows the cattle producer to make long-term and near-term plans to minimize risk. Veterinarians can help beef cattle producers take appropriate actions to prevent neonatal calf losses by conducting a herd-specific risk assessment.

Risk assessment is a process of:

1) evaluating the likelihood and costs (or benefits) of potential hazards (or opportunities), termed risk analysis;
2) determining what actions, at what relative cost, can be taken to mitigate those hazards, termed risk management; and
3) sharing the action plan with all members of the team, as well as keeping records to show what was done and whether the actions were successful, termed risk communication.

The process of risk assessment can be iterative to provide a continuum of actions toward ongoing improvement [1]. A risk assessment may be completed formally or informally, and the process may be qualitative or quantitative [2, 3]. As part of the risk analysis phase it may be necessary, or at least useful, to supplement published evidence with herd-specific data from health records [4], outbreak investigation [5], or clinical trials [6]. It is possible to recognize important hazards and estimate their costs without quantitative data during the risk analysis stage, but it is more difficult to evaluate progress or compliance in the risk management stage without using records.

Unfortunately, few cow-calf operations collect animal health data in a format that is easily analyzed [7]. The lack of a simple record-keeping system on many farms hinders the process of recognizing important hazards and their costs, and makes it difficult to document that risk management actions were implemented or to evaluate if those actions were effective. If records exist at all, they may be on paper (e.g. in pocket-sized health record books) or in a computerized digital format (e.g. spreadsheets or databases) with free text fields, none of which allow the veterinarian or the producer to quickly and easily discover animal health relationships [7]. Some proprietary database systems capture health information and may present information in standardized graphs or summaries, but do not always allow easy querying of other health relationships. Free text fields are difficult or impossible to analyze because of misspelled words, multiple names or descriptors for the same disease (e.g. diarrhea, scours, loose, enteric), and multiple pieces of information in the same field. Often it is too costly to wade through inefficient record systems to get the needed information from which a risk assessment can be performed. Therefore it may be beneficial for the veterinarian to help the cattle producer establish a system for capturing and analyzing health and performance data.

A desirable system for evaluating calf health and performance captures data on all calves at risk, not just treated calves. Useful information includes the calf's identification, identification of the dam, age of the dam, calf's birth

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

date, date of onset of health concerns, and information describing variables of interest (e.g. birth and weaning weights, risk factors for disease, economic data).

A risk assessment evaluates the reasons for the occurrence of hazards, the likelihood of the hazard, and its cost. In the absence of farm-specific information, risk assessments are often based on published information and expert opinion. For example, based on a national survey of beef cattle herds completed in 2007–2008 [7], 2.9% of calves were born dead and another 3.5% died or were lost prior to weaning. These rates were similar, regardless of the herd size. In this survey, the reasons for beef calves to die in the first three weeks of life, in order of frequency, were (i) birth related (25.7% of deaths); (ii) weather related (25.6%); (iii) unknown causes (18.6%); (iv) digestive system related (14%); (v) respiratory disease (8.2%); and (vi) predation or injury (6.2%). Note that these are population averages and not every beef herd experiences losses according to this distribution. However, in the absence of herd-specific information, these data inform us that, on average, the important hazards to the survival of neonatal calves are problems occurring during calving, dangers from the environment, and contagious diseases.

Managing risks requires greater understanding of the factors associated with those hazards, how to mitigate those factors, and the associated costs [1]. Economics should not be the sole basis for making decisions about the care of animals. However, the cost of healthcare remains an important financial constraint to most cattle producers and is therefore an important consideration. The relative costs of disease prevention and control practices are relevant to risk management decisions. Comparing the relative costs of different approaches to managing risk can help inform animal care decisions.

The cost of even highly effective risk management practices should not be overlooked. As a simplified example, assume that a vaccine is 75% effective and costs $10 per head to administer (e.g. for product, labor, and supplies). If the disease occurs at an annual incidence of 40 cases per 10 000 head, then vaccination would reduce the incidence to 10 cases per 10 000 head per year. The annual cost to prevent 30 cases in a population of 10 000 would be $100 000, or $3333 to prevent each case. In this scenario, use of the vaccine might only be economically justified to prevent extremely costly diseases (e.g. causing high mortality in herds of high value). If the annual incidence of disease were 400 cases per 10 000, the cost of preventing each case would be $333. In this scenario, if the cost of the disease (e.g. costs for labor to treat, medications, lost performance, and deaths) averaged $100 per case, then use of the vaccine would not be economically justified unless the expected incidence in the absence of vaccine was 1333 cases per 10 000.

Factors other than direct monetary costs or benefits play into a farmer's decision to use a preventive intervention. It might be more useful to consider units of utility rather than units of money in the calculation of risk management costs and benefits [1]. For example, in addition to the animals' monetary value, a particular farmer's unit of utility might also include their concern for the health and welfare of their cattle or pride of owning healthy and productive cattle. Also, average or expected values do not consider the impact of extreme events. For example, the occurrence of infectious diseases may cluster in time and place as an outbreak. Therefore, just as with many insurance plans, the decisions to use vaccines or to take other preventive actions may be based on minimizing the risk of a catastrophic outbreak, even if the average cost of the prevention is greater than the average cost of the disease. Finally, the relative level of risk aversion of the individual producer may affect the utility breakpoint at which a risk management practice is adopted [1].

What follows are qualitative risk assessments with generalized approaches to managing the risk of the most common hazards of the neonatal period in beef herds. The veterinary practitioner may be able to provide a more tailored approach to managing health risks within specific cattle herds using specific herd data. The emphasis of this chapter is on disease and injury prevention; however, timely and effective treatment is also important in managing the overall cost of disease or injury.

Risk Assessment of Calving Hazards

Successful calving occurs when a live calf is born without complications to the calf or the dam. Problems with the birthing process are called dystocia. Dystocia may be due to factors of the calf or factors of the dam [8].

Calf Factors of Dystocia

The factors of dystocia related to the calf are size, posture, and presentation. Large calves are more likely to experience dystocia because they have difficulty fitting through the dam's pelvis. Posture refers to the position of the calf's head and legs as the calf enters the birth canal. Presentation refers to the calf's orientation relative to the dam. The most normal presentation and posture at birth is the calf upright, facing toward the rear of the cow, with the head between extended front legs. A breech birth is an example of both abnormal posture and presentation; the calf is backwards with the rear legs folded under the body. Of these factors, calf size is the most common problem and most preventable, through genetic selection [8].

The consequences of dystocia to the calf are metabolic or physical injury that may result in death during or following the birthing process. Lack of oxygen in the blood causes injury to cells and results in acidosis and low blood sugar. Physical injuries include congestion and swelling of the head and tongue which may prevent nursing, or broken bones due to excessive force during calving assistance.

Dam Factors of Dystocia

Factors of dystocia attributable to the dam are age, pelvic size, and metabolic health [8]. Dystocia is more common among heifers and also cows with small pelvic dimensions. Common metabolic problems at calving are from muscle weakness due to protein–energy malnutrition, exhaustion during prolonged muscular contractions, and low blood levels of calcium or magnesium. The dam may experience metabolic or physical injury during or following the birthing process. The most common problems for the dam are exhaustion from muscular contractions, pressure injury to leg muscles while being recumbent, and bruises or tears to the uterus and vagina. The consequences of these problems include failure of the dam to get up after calving, prolapse of the uterus, excessive bleeding, or infection of the reproductive tract. Each may ultimately be fatal.

Managing the Risk of Dystocia

There are long-term and near-term strategies for preventing problems associated with dystocia [9]. Long-term strategies include genetic selection for calving ease and pelvic size, and implementing sound heifer breeding and development programs. Near-term strategies include providing exercise and balanced nutrition to heifers and cows prior to and after calving, frequent monitoring of calving progress, early and appropriate calving assistance, ready access to the appropriate tools for calving assistance, copious use of lubricants, and attention to sanitation (e.g. use of soap and water) during birthing assistance. Describing methods for calving assistance are beyond the scope of this chapter; however, an important aspect of managing dystocia risk is helping producers know when veterinary assistance should be sought. Cattle producers should seek veterinary assistance when they:

- do not know what is wrong;
- know what is wrong, but either do not know what to do or recognize it is beyond their abilities; or
- know what is wrong and what to do about it, but have been unsuccessful after 30 minutes of trying [10].

Risk Assessment of Environmental Hazards

The environmental conditions in the neonatal period are important to the health and well-being of the dam and her calf.

Environmental Hazards to the Calf

Common environmental factors that present a hazard to the neonatal calf are weather extremes, crowding, predators, and physical sources of injury. At birth, the calf is limited in its ability to regulate its body temperature, so extremely warm or cold environmental conditions present a risk for hyperthermia or hypothermia, respectively, especially when accompanied by wet and muddy or dry and dusty conditions. Crowded herd conditions increase the chance for injury from being stepped on, butted, or otherwise injured by others in the herd, and increase opportunities for pathogen exposure and transmission. Predators present the greatest hazard to calves recently born or calves weakened by illness or injury. Calves may be injured from a variety of physical hazards in the calving environment including protruding nails, broken posts, loose wire, holes, steep embankments, standing water, various sources of electricity, and toxins (e.g. from lead batteries or chemical containers).

Environmental Hazards to the Dam

Cows are less susceptible to weather stressors compared to their calves, but dystocia or metabolic disease increases their risk for hypothermia or hyperthermia. During the periparturient period, cows may be more likely to slip and fall, and the likelihood further increases when the floor surface has a steep slope or is slippery due to snow, ice, or mud. Cows calving near ditches and streams or other low spots are at risk to fall or not be able to rise after lying down. As with calves, cows may be injured by a variety of physical hazards that may be present in the calving environment.

Risk Management of Environmental Hazards

The risk of injury to cow or calf can be minimized by providing favorable environmental conditions. Long-term strategies include breeding in a time frame such that calving occurs during favorable weather conditions and designing and using calving facilities with minimal physical hazards. Near-term strategies for environmental safety include a precalving survey of the environment and facilities for potential sources of injury, and frequent surveillance of the herd during the calving season.

Risk Assessment of Neonatal Calf Diarrhea

Neonatal calf diarrhea, commonly called scours, is one of the most likely reasons for morbidity or mortality of beef calves [7]. Neonatal calf diarrhea is a detriment to calf health and well-being, and the disease is costly to cattle producers due to reduced calf growth performance, death loss, and the expense of labor and medicines to treat sick calves [11, 12]. Also, restraint and treatment of calves puts veterinarians, herd owners, and their employees at risk for injury or zoonotic infection. Finally, it is disheartening to most cattle producers to see young calves sicken or eventually die following often valiant, but futile, treatment attempts.

Neonatal calf diarrhea is a complex disease [13–16], with many interrelated component causes. Component causes are the factors that in combination are sufficient to cause signs of disease [17]. The component causes of neonatal diarrhea in calves include more than the etiologic agent. In fact, it may be rare that knowledge of the presence of an agent by itself leads to a solution to outbreaks of neonatal calf diarrhea. Agent, host, and environmental factors collectively explain the occurrence of clinical signs of diarrhea, and these factors interact dynamically over the course of time [18]. To make effective risk management decisions, cattle producers and their veterinarians must understand the dynamic relationships occurring between agent, host, and environmental factors within the context of the specific production system [19]. For example, even if the etiologic agent(s) involved are known, understanding the various sources of the agent and routes of transmission on the farm might be useful for prevention and control.

Agent Factors of Neonatal Calf Diarrhea

Numerous pathogens have been recovered from cases of neonatal calf diarrhea, including bacteria such as *Escherichia coli* and *Salmonella* spp., viruses such as rotavirus and coronavirus, and protozoa such as cryptosporidia [13, 14, 19–26]. The adult cow-herd commonly serves as the source of pathogens from one year to the next [27–32]. The usual pathogens associated with neonatal calf diarrhea can commonly be recovered from calves in most cattle herds, including calves in herds of cattle not experiencing calf diarrhea [19]. Recovering a pathogen from a calf with diarrhea might help to explain the immediate cause of the calf's morbidity or mortality. However, that knowledge alone rarely explains the reason for the outbreak, nor does it necessarily provide a complete approach to treatment, control, or prevention of cases [16]. Multiple agents can often be recovered from calves in herds experiencing outbreaks of calf diarrhea, suggesting that, even during outbreaks, more than one agent may be involved.

Host Factors of Neonatal Calf Diarrhea

After absorbing antibodies from colostrum or colostrum supplements in the first 24 hours of life, calves may obtain passively acquired maternal immunity against diarrhea-causing pathogens common to the herd [33–35]. The quantity of antibodies absorbed is determined by the quality and quantity of colostrum the calf ingests, how soon after birth it is ingested, and the calf's physiologic state [34, 36]. The presence of antibodies in colostrum requires prior exposure of the dam to the wild-type agent or vaccine antigens. Vaccines are sometimes used to immunize the dam against specific agents so that the calf will receive passive antibody protection from colostrum [16, 37]. Also, some commercially available colostrum supplements and replacers contain antibodies directed against specific calf diarrhea pathogens [38, 39]. Unfortunately, the use of vaccines or colostrum supplements has not always prevented outbreaks of calf diarrhea in beef cattle herds.

Calves become ill or die from neonatal diarrhea within a narrow range of age, typically one to three weeks of age [13, 20, 22, 40]. The narrow range of age within which calf diarrhea occurs cannot be explained by the incubation period of the agents because diarrhea is observed within a few days of experimental pathogen challenge in colostrum-deprived or gnotobiotic calves, regardless of age [41–43]. It may be that calves experience an age-specific susceptibility to diarrhea agents that occurs as maternal immunity is waning and before the calf is primed for an effective immune response [37]. Therefore the first 7–21 days of age typically define the age of susceptibility, as well as the age calves are most likely to initially shed diarrhea-causing pathogens in their feces, becoming infective to other calves in the herd [44–48]. In some calving systems, the relative number of susceptible and infective calves can change dynamically with time [18]. Exposure to pathogens of a dose-load or duration sufficient to cause disease is called an effective contact. At times, particularly late in the calving season, the number of infective calves may greatly outnumber the number of susceptible calves, resulting in widespread opportunities for effective contacts.

The dam's age also contributes to a neonatal calf's risk for diarrhea. Calves born to heifers often have a higher risk for diarrhea and have lower maternal antibody levels than calves born to older cows [49]. Calves born to heifers are probably more susceptible to disease in the neonatal period because heifers produce a lower volume and quality of colostrum, may have poor mothering skills, and are more likely to experience calving difficulty [50, 51].

Environmental Factors of Neonatal Calf Diarrhea

Environmental conditions may contribute to both the level of pathogen exposure and the ability of the calf to resist disease. Exposure to pathogens may occur through direct contact with other cattle or via contact with contaminated environmental surfaces. Keeping the environment clean has long been recognized as important for preventing calf diarrhea [52, 53], but it may not be possible to do so effectively. Crowded conditions increase opportunities for effective contacts with infected animals or contaminated surfaces. Ambient environmental temperature (e.g. excessive heat or cold) and moisture (e.g. mud or snow) are important stressors to the neonatal calf, may impair the calf's resistance to disease, and also affect pathogen concentration in the environment, opportunity for exposure to pathogens, and dose-load of ingestion.

Temporal Factors of Neonatal Calf Diarrhea

Host susceptibility, pathogen exposure load, and pathogen transmission occur dynamically over time within the calving season [19]. Although the adult cow-herd likely serves as the source of most calf scour pathogens from year to year [27–32], the average dose-load of pathogen exposure to calves is likely to increase over time within a calving season because calves infected earlier serve as pathogen multipliers and become the primary source of exposure to younger susceptible calves. This multiplier effect can result in high prevalence of infective calves and widespread environmental contamination with pathogens [54]. Each calf serves as growth media for pathogen production, growing the number of pathogens to even greater numbers [43–45]. Therefore calves born later in the calving season may receive larger dose-loads of pathogens and, in turn, may become relatively more infective by growing even greater numbers of agents. Eventually the dose-load of pathogens overwhelms the calf's ability to resist disease. These factors alone or in combination may explain observations that calves born later in the calving season are at greater risk for disease or death [18].

Risk Management of Neonatal Calf Diarrhea

Biosecurity is the sum of actions taken to prevent the introduction of a disease-causing agent into a population (e.g. pen, herd, or region) and biocontainment describes the actions taken to control a pathogen already present in the population [55]. In theory there are three approaches to preventing outbreaks of neonatal calf diarrhea: (i) eliminate the pathogens from the population; (ii) increase calf immunity against the pathogens; or (iii) alter the production system to reduce opportunities for pathogen exposure and transmission [56]. However, the endemic nature of the common pathogens of calf diarrhea makes it difficult or impossible to make cattle populations biosecure from these agents. Colostral immunity is critical to protect neonatal calves from disease [37]. However, passive immunity against diarrhea pathogens decreases with time [37], and managers of beef cattle herds have limited ability to improve calf ingestion and absorption of colostral antibodies beyond not interfering with maternal bonding. Unfortunately, vaccines are not available against all pathogens associated with calf diarrhea, vaccines may not provide sufficient cross-protection against all pathogen strains [48], and pathogens may evade the protection afforded by vaccination by evolving away from the vaccine strains [57]. For these reasons, a biocontainment approach for controlling calf diarrhea, primarily by reducing effective contacts, may be a useful risk management strategy in many cattle production systems [56].

Effective contacts can be prevented by physically separating animals, reducing the dose-level of exposure (e.g. through the use of sanitation or dilution over space), or minimizing contact time [56]. For example, these principles have been successfully used to prevent neonatal illnesses in dairy calves by using calf hutch systems [58]. Various biocontainment systems for beef herds have been developed to prevent calf diarrhea [59–62]. Each of these strategies is designed to manage cattle in a system that prevents calves from having effective contacts with pathogens by reducing opportunities for exposure and minimizing dose-load.

One example of a beef cattle biocontainment system for controlling neonatal calf diarrhea is the Sandhills Calving System [62, 63]. The management actions defined as the Sandhills Calving System prevent effective contacts among beef calves by segregating calves in calving pastures by week of age. This is achieved through scheduled weekly movement of pregnant cows to clean calving pastures. The objective of the system is to recreate, during each subsequent week of the season, the more ideal conditions that exist at the start of the calving season, where cows are calving on ground that has been previously unoccupied by cattle (for at least some months) in the absence of older, infective calves.

The Sandhills Calving System prevents effective contacts by using clean calving pastures, preventing direct contact between younger calves and older infective calves, and preventing later-born calves from being exposed to an accumulation of pathogens in the environment. The specific actions to implement the system may differ between herds in order to meet the specific needs of each production system. Key components of the systems are age segregation of calves, the frequent movement of pregnant cows to clean calving pas-

tures, and opportunity for maternal bonding and colostrum ingestion with little management interruption. Age segregation prevents the serial passage of pathogens from older calves to younger calves. The routine movement of pregnant cows to new calving pastures prevents the build-up of pathogens in the calving environment over the course of the calving season and helps to prevent exposure of the latest-born calves to an overwhelming dose-load of pathogens.

Ranchers using the Sandhills Calving System have observed meaningful and sustained reductions in morbidity and mortality from neonatal calf diarrhea, and greatly reduced use of medications [63]; however, there may be additional costs associated with this production system compared with other methods of calving management. For example, there may be changes in land usage that require fencing or placement of water sources. Time and labor devoted to checking cows and delivering feed may increase. Cattle calving in extensive pastures may have greater health risks due to inclement weather or unobserved dystocia. Development of a ranch specific plan for implementing the Sandhills Calving System or other biocontainment system must take place well in advance of the calving season. Available pastures must be identified and their use coordinated with the calving schedule. Water, feed, shelter, and anticipated weather conditions must be considered. The size of the pastures should be matched to the number of calves expected to be born in a given week so as not to be damaging to later grazing.

The costs of implementing the Sandhills Calving System may be offset by benefits to labor management. For example, there may be some efficiency because cattle movement could be scheduled once a week as labor is available. Moving cows without calves to a new pasture is often easier than sorting and moving individual cow-calf pairs. Also, the workload is partitioned between pasture groups such that cows at risk for dystocia are together in one pasture while calves at risk for diarrhea are in another.

Risk Assessment of Pneumonia in Calves Prior to Weaning

Pneumonia is the leading cause of death in calves from three weeks of age to weaning [64]. This is perplexing considering that, before weaning, calves typically live in conditions with little stress and relative isolation from external sources of pathogens. And yet, preweaning pneumonia affects approximately 20% of beef cow-calf herds [65, 66]. Pneumonia in calves prior to weaning may affect up to 10% of US beef calves [67], resulting in death of 0.6–1.4% of all calves [68–70]. Calves affected with preweaning pneumonia may weigh 17–37 pounds less at weaning compared to unaffected calves [70, 71]. Pneumonia in calves prior to weaning is typically seen as one of two syndromes [72]. The first is intermittent, low incidence pneumonia in calves less than one month of age; the other is as a rapidly occurring outbreak of pneumonia in a large portion of calves in the herd when calves are three to five months of age. This latter syndrome is paradoxically associated with characteristics of highly managed herds, including having a larger herd size, using rotational grazing, and practicing estrus synchronization [65, 66]. It is likely that these characteristics are associated with greater opportunities for effective contact between calves. The annual cost of pneumonia in beef calves prior to weaning to the US beef industry is approximately $165 million [73]. The cost of labor to treat pneumonia in beef calves often exceeds the cost of medication [74].

Agent Factors for Preweaning Pneumonia

The common bacterial pathogens of pneumonia, *Mannheimia haemolytica*, *Pastuerella multocida*, and *Histophilus somni*, are found in the upper respiratory tract of most cattle. The inciting damage is often due to viral infections that may not be present in all cattle herds all the time. The most commonly recognized viral pathogens are bovine herpes virus 1, bovine viral diarrhea virus, and bovine respiratory syncytial virus, but many others are likely to be involved, including bovine coronavirus [75, 76]. Invasion of the lower respiratory tract with a pathogen may be a necessary condition of pneumonia, but exposure to these pathogens is not causally sufficient because it is difficult to create pneumonia through experimental challenge with bacteria or viruses alone [77].

Host Factors for Preweaning Pneumonia

Passively acquired maternal antibodies, important for protecting young calves against respiratory pathogens, wane with time. The serum half-life of passively acquired immunoglobulin G (IgG) is approximately 16–20 days, so that by the time a calf is 90–120 days of age, less than 2% of the antibodies it absorbed from ingested colostrum remain. The immune system is functional but unprimed at birth and acquired immunity is weaker, slower, and more easily overcome prior to five to eight months of age [78]. Therefore calves that are three to five months of age may be particularly susceptible to pneumonia even in the absence of additional stressors.

Environmental Factors for Preweaning Pneumonia

Although the setting for cow-calf production is often extensive and pasture-based, the opportunities for pathogen transmission from calf to calf may be relatively high because, regardless of the size of the pasture, cattle are gregarious and

will congregate closely around water sources and feedbunks, in shade, and when bothered by flies. Some management practices such as pasture moves and gathering for sorting also result in temporary periods of high animal density and greater opportunity for pathogen transmission.

Temporal Factors for Preweaning Pneumonia

Intermittent pneumonia in very young calves is likely due to calves with failed transfer of passive immunity [72]. On the other hand, it is the age-related loss of maternal immunity that may explain sudden outbreaks of preweaning pneumonia in herds with managed breeding seasons [72]. Herd immunity is the protection afforded to susceptible individuals because most of the individuals in the population are immune [79]. In herds with a narrow window of calving, most calves are relatively similar in age and herd immunity may be lost over a relatively short span of time as the majority of calves in the herd approach 90–120 days of age. Other temporal factors, such as weaning, commingling of groups, and severe weather can be important stressors and further reduce a calf's ability to resist disease.

Risk Management of Preweaning Pneumonia

Actions that increase the opportunity for calves to obtain colostrum in the first few hours after birth reduce the risk for failed transfer of passive immunity and may reduce the risk for pneumonia in young calves. It seems prudent to take actions to reduce the rate of effective contacts in herds that regularly experience outbreaks of pneumonia in calves three to five months of age. Also, vaccination of calves against bovine respiratory disease prior to three months of age seems prudent and has been anecdotally effective, although logistically challenging. However, the optimum set of protocols to prevent pneumonia in calves of this age remains an important subject of investigation.

Summary

Beef cattle producers can anticipate the most likely and most costly hazards to their cows and calves at calving time and can use this knowledge to plan for their prevention. Minimizing the risk of these hazards requires long-range and near-term planning. Calving problems may occur because of factors of the calf or the dam. Planning ahead for calving problems and close monitoring of the herd during calving can minimize the likelihood and/or cost of dystocia. Environmental conditions such as weather or physical hazards in the calving area are also important sources of injury to cows and calves. Planning to calve during favorable weather seasons and monitoring the environment for dangerous conditions minimizes the risk of these hazards.

A common cause of sickness or death of calves in the first three weeks of life is diarrhea. Understanding the complex interactions that cause calf diarrhea is the basis for developing strategies for disease control and prevention. The common pathogens of calf diarrhea are common to most cattle herds and it is unlikely that cattle could be made biosecure from these agents. Managers of beef cattle herds have few opportunities to improve rates of colostrum uptake and absorption, and vaccines are not always protective. Colostral immunity wanes, making calves age-susceptible and age-infective. Each calf serves as growth media for pathogen production, amplifying the dose-load of pathogen it receives and resulting in high calf infectivity and widespread environmental contamination over time in a calving season. For these reasons it is logical to apply biocontainment strategies to prevent effective transmission of the pathogens causing diarrhea. Cattle management systems based on an understanding of infectious disease dynamics have successfully reduced sickness and death due to calf diarrhea.

The most common cause of death in calves from three weeks of age until weaning is pneumonia. As with neonatal calf diarrhea, loss of maternal immunity may play a role in outbreaks of pneumonia, especially in calves three to five months of age. Outbreaks of pneumonia in beef calves have, paradoxically, been associated with characteristics of highly managed herds that create more opportunities for effective contacts. Strategies to prevent or control outbreaks of pneumonia in calves prior to weaning are still a subject of investigation.

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Management to Decrease Neonatal Calf Loss in Dairy Herds

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Introduction

The neonatal life, between birth and 30 days of age, is known among dairy producers and veterinarian practitioners to be determinant and most challenging for the calf as it is the time period when the greatest mortality occurs. The ultimate objective of all dairy farmers is to raise healthy and productive replacement dairy heifers. This chapter emphasizes practical approaches for decreasing morbidity and mortality of dairy heifers from birth to weaning, focusing on topics such as the pregnant dam, the calving period, neonatal dietary requirements, husbandry, and health maintenance of the newborn dairy heifer calf.

From Birth to Weaning

Neonatal morbidity and mortality not only can be influenced by the nutritional and physiological status of the dam but also may be directly associated with genetic traits [1]. Calf sire and breed [2], dam breed, trait heritability, feed efficiency, milk yield, carcass composition, and gestation length [3–5] are some examples of genetic variabilities that will in some degree influence neonatal death [1]. Calving season, calving environment, dry cow nutrition, dam vaccination history, and other specific risk factors associated with dystocia [6] such as age at first calving [2], gender and birth weight [3, 7], stress and uterine inertia, as well as infectious and non-infectious causes following eutocia such as congenital defects [8], twinning [7, 9], prematurity or dysmaturity, and abnormal placentation or premature placenta separation [10] are some non-heritable traits that may lead, as one factor or multifactorial reasons, to the demise of the calf during or shortly after calving.

The producer with the help of the veterinarian practitioner should always explore and analyze the situation by becoming aware of the risk factors for the individual or more importantly the cohort of animals where the problem has occurred. Proper animal identification and knowledge of the reproductive status of the dam are imperative for a successful disease investigation.

European researchers [11] published in 2016 a review on the influence of epigenetics and fetal programming on certain traits of production animals. They detailed the impact of the in utero environment and its influence on the ruminant progeny's overall health and welfare. It is suggested in the literature that fetal programming in the neonate during pregnancy may occur by epigenetic changes that are controlled by gene expression in the placenta and fetus (see Chapter 27, "Fetal Programming").

The maternity area or pen should have good lighting, with minimum human and farm equipment traffic, and be separate from the rest of the herd; also producers should avoid multiple-use areas as these can serve as focal points to spread infectious agents and transfer diseases between newborns and adults. A well-designed facility does not replace good management, and thus close observation is essential so that the cattle spend the minimum necessary amount of time in the calving area. It is imperative to note that if cows are to be calved in a designated pen or stall, providing a well-bedded dry enclosure with good air quality is essential. Larger dairies may opt to use calving paddocks or pastures, which allows close observation and therefore prompt movement of cows close to calving (two to three weeks prior to calving) and fresh cows.

The economics associated with raising a dairy heifer are positively related to the price of feed (milk, milk replacer, waste milk and pasteurization, concentrate), type of housing, death losses, culling rate, and other fixed variable

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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costs [12, 13]. Zwald et al. [13] reported that the average cost of raising a replacement heifer in the northern USA was about $4.28 per day from birth to moving the heifers out of hutches into group pens. A more recent report published by Iowa State University Extension and Outreach in 2019 [14] suggested an increase over the past years of roughly $1.00–1.25 per calf per day from birth to weaning. This means an estimate for a typical 56-day birth to weaning period is now a little over $300 in total cost per calf. The same publication notes that a good rule of thumb to remember is that overall cost until breeding age, in 2019, was approximately $2.33 per head per day for a 700-pound heifer. Further, to get the heifer to two years of age, the report indicated that after accounting for the extra costs associated with reproduction and labor, a typical $2.67 per head per day would be realistic considering the region and current economy as a whole.

Colostrum

Colostrum management is critical: for neonates to achieve successful transfer of maternal immunoglobulin (Ig), a sufficient amount of immunoglobulin must be present in the colostrum, combined with a low bacterial count [measured in colony-forming units (cfu)], and be consumed by the calf shortly after birth while the large immunoglobulin molecules are still able to be absorbed through the intestinal mucosa. Colostrum management is discussed in Chapter 75.

High-quality colostrum contains immunoglobulin at a concentration in excess of 50 g/l with a bacterial count of less than 100 000 cfu/ml [15]. Every neonate should be ingesting a minimum of 150–200 g of immunoglobulin within the first four hours of life [16]. As far as the volume to be offered, the rule of thumb is 3–4 l of good-quality (>50–75 g/l of IgG) colostrum; however, one must also consider the 10–12% of body weight rule [17] such that overfeeding should be avoided in some lighter calves, such as Jerseys and low-birthweight Holsteins, and some of the dual-purpose breeds (e.g. Milking Shorthorn, German Fleckvieh). It is important to note that genetics, production levels, and dry period length [18] positively impact the quality of colostrum.

For optimum health, calves less than one week old that received sufficient good-quality colostrum should manifest minimum serum IgG_1 concentrations of 1 g/dl [19]. High coliform-type bacterial count may bind free Ig in the intestinal lumen and/or directly block uptake and absorption of the Ig molecules by the epithelial cells, thus interfering with passive transfer. Recent work by Elizondo-Salazar and Heinrichs [20] showed that the best method to reduce bacterial counts in colostrum is to batch pasteurize at 60 °C for 60 minutes, known as the 60/60 rule. This treatment has been proven to reduce the concentration of pathogenic bacteria while preserving the nutrient value and provide a superior-quality Ig to the neonate. The shelf-life with refrigeration is 8–10 days [17].

Nutrition from Birth to Weaning

Drackley [21] published a comprehensive report on calf nutrition in 2008. To summarize, a 45-kg calf requires 1.75 Mcal/day for maintenance in a thermoneutral environment. This is equivalent to 325 g of milk solids or 2.5 l of whole milk or 3 l of milk replacer, as the latter is lower in fat content compared with whole milk. As a guideline, producers should be feeding calves a milk diet from birth to weaning at a measured quantity of 10% of body weight per day, divided into two feedings; this amount may be adjusted up to 12% during the winter months [22]. Increasing the frequency of feeding (three or four times a day), increasing volume (15% of body weight a day), and increasing the solid contents are some techniques producers may use to increase weight gain. Obviously there are disadvantages associated with those decisions, as increasing volume and frequency may decrease starter grain intake and cause digestive upset and diarrhea and the total solids in milk replacer should not exceed 15%.

Another factor to take into consideration is the type of milk replacer and the fat/protein concentration. Typically, 20 : 20 is most commonly found; however, one must consider the season, as winter months require more energy, cost, and overall health performance (e.g. due to presence of diseases, weight loss vs gain). Traditional versus accelerated feeding programs of dairy calves are some strategies often used by the dairyman. The traditional program uses 20% protein and 20% fat, as mentioned above. Often energy intake and growth are limited due to the levels fed. Higher intake may give calves enough nutrition for growth, but the higher protein will limit gain. The accelerated method uses milk replacers with higher protein (up to 28%) and lower fat (~15%) content. This feeding scheme tends to be more costly, and the producer must look at the season, as lower fat during the winter is not desirable.

Often it is more economical to feed whole milk (out of the bulk tank) instead of replacer; if that is the case, pasteurization is highly recommended to decrease bacterial count and cow–calf disease transmission (e.g. paratuberculosis, salmonellosis).

Calf starter, commonly called calf manna, provides another source of energy for calves and it can be offered as early as 1.5–2 weeks of age along with milk. An amount of

1.5% of body weight for the first week after birth increasing to 2% until weaning (10–12 weeks old) is recommended. It is also important to note that a calf should not be weaned off milk completely until it is regularly consuming 1 kg of starter feed daily [21]. The dairyman should always consult with the veterinarian of record and the nutritionist to determine an appropriate feeding scheme and type of milk replacer program.

Calf Housing

It is commonly understood among dairy producers, staff, and veterinarian practitioners that all calves must be kept dry, free from drafts, and separate from each other (no nose-to-nose contact) until weaning. The health advantages associated with this type of husbandry are well described in the literature [22–24].

Calf hutches or individual pens have been standard in the dairy industry [25] for many years. A standard minimum dimension of 3 m^2 is recommended and about an extra 10–15% open area between calves must be available to allow for cleaning and disinfection [20]. Different calf hutches/pens are available on the market and their use will vary depending on the region of the USA. Calf barns are very popular in the northern region, whereas outdoor hutches are commonly found in other areas of the USA. Hutches are usually not bedded (slatted wood floors, California-style hutches), or bedded with sand, straw, shavings, or other suitable material. The author recommends the use of sand as bedding due to its inorganic characteristics which facilitates disease prevention by offering the best when it comes to drainage of urine and feces. The calf hutch should face south in the winter and north in the summer [22], and be located in a prepared, sloped area to allow water drainage away from the hutch. Dirt, gravel, concrete, or slatted wood may be used as flooring material. A rack to hold water and feed buckets and/or nipple bottle should be placed outside the hutch, as this significantly reduces bedding maintenance. Common biosecurity practices must be practiced daily. Nipple bottles and water and feed bucks must be properly disinfected and rinsed, and must not be swapped between calves.

Weaning

Although age (10–12 weeks), weight (90–100 kg), and health condition must be considered, a calf should be weaned off milk and moved into a group pen only when consuming at least 1 kg of solids for three consecutive days [21, 22]. It is advisable to keep the calf in the hutch up to 10 days with starter feed ad libitum after complete removal of the milk diet; this practice allows the calf to lose its urge to nurse, as well as encouraging the consumption of dry feed on a regular basis, adapting and promoting rumen growth. Vaccination against the most common respiratory diseases may be considered before moving into the group pens. The author's experience suggests the use of a commercial intranasal product used according to the label and with the veterinarian on record's consent.

Health Maintenance

As discussed previously, good-quality colostrum delivered to the neonate within the first four to six hours after birth is essential for successful passive transfer of maternal immunoglobulins. It is recommended practice to remove the calf to a clean and dry area, protected from severe weather conditions, where basic calf care can be done. Close examination for any obvious gross abnormalities, such as cleft palate or other birth defects, followed by inspection and disinfection of the umbilicus are critical. Proper identification using tags and the placement of an official United States Department of Agriculture (USDA) identification, radio frequency identification tags (RFIDs), and maintenance of accurate records are also important (livestock owners should follow their state rules). The use of a "calf card" with information such as date of birth, sex, dam number, colostrum feeding (amount and quality), calving ease, and general comments is recommended. This information can be easily transferred to the dairy management software for easy access at a later date.

A comprehensive review article summarized the role of vaccines and of the neonatal immune system, as well as its impact on vaccine response [26]. The authors concluded that, first and foremost, one must assess the disease risks at the production site, consider the dam's immunity (i.e. colostrum quality), and the age of the calf in order to determine the vaccination protocol. Despite new research being done to avoid vaccine interference with maternal antibodies, one must never undervalue the importance of delivering good-quality colostrum, nor support the extra-label drug use of vaccines [27]. Vaccination schedules are farm specific and vary according to dairy practices; however, an example of a classic timetable is summarized in Table 73.1.

The most recent National Animal Health Monitoring (NAHMS) Beef 2017 Cow-Calf Study [28] reports 7.8% of beef cattle in the USA are horned, improved from 27.8% horned in 1997 and 12.4% in the 2007–2008 survey; conversely, NAHMS Dairy 2014 [25] reported that roughly 95% of dairy operations dehorned or disbudded heifer calves

Table 73.1 Typical vaccination protocol. Variation and inclusion of other pathogens (rotavirus, coronavirus, *Moraxella bovis* and *bovoculi*, etc.) may vary among farms.

Pathogen	Route	Age at first dose	Booster (follow label)	Comments
IBR	sc or in	6–8 weeks	3–4 weeks later	Revaccinate at breeding age (10–12 months old), then yearly
PI3	sc or in	6–8 weeks	3–4 weeks later	Revaccinate at breeding age (10–12 months old), then yearly
BRSV	sc or in	6–8 weeks	3–4 weeks later	Revaccinate at breeding age (10–12 months old), then yearly
BVD (types 1 and 2)	sc or in	6–8 weeks	3–4 weeks later	Revaccinate at breeding age (10–12 months old), then yearly
Clostridium spp.	sc	6–8 weeks	3–4 weeks later	Revaccinate at breeding age (10–12 months old), then yearly. Inactivated toxoid, local reaction may occur
Leptospirosis (five-way)	sc	6–8 weeks	3–4 weeks later	Revaccinate at breeding age (10–12 months old), then yearly. May consider including hardjo-bovis strain

Must follow label directions and the veterinarian on record recommendations.
Follow the vaccine label with respect to indication, dose, route, frequency, and meat withdrawal.
IBR, Infectious bovine rhinotracheitis; PI3, parainfluenza 3; BRSV, bovine respiratory syncytial virus; BVD, bovine viral diarrhea; in, Iintranasalntranasal; sc, subcutaneous.

and more than half of those procedures were accomplished by the producers and not by a veterinarian.

In November 2019, guidelines regarding dehorning of calves were published by the American Association of Bovine Practitioners [29] to help veterinarians and producers standardize this common livestock procedure to enhance overall knowledge of what and how this procedure is performed, subsequently enhancing animal welfare. It is widely known that by having non-horned cattle, the risk of injuries to herd mates and farm personnel is greatly reduced, as well as affording easier use of milking facilities, animal handling areas, and feed and water trough areas. A variety of methods are available and choice commonly differs by age of the calf at time of the procedure.

Chemical caustic paste, Barnes or scoop-type instrument, electric or gas-filled iron, or a combination of Barnes and dehorning iron are the options available [30]. The author suggests this procedure is done as early as possible in life. Removing the horn bud (while the bud is not yet attached to the skull, usually by eight weeks of age) is a lot easier and less traumatic to the patient.

The application of caustic paste is advantageous if applied correctly over the horn buds and in calves that are less than two days old, housed individually, and protected from a wet environment [31]. The use of an electric or butane gas-filled dehorning iron at one to four weeks of age is the quickest and most efficacious method for removal of the horn bud [22, 31, 32]. The procedure is simple but requires precise restraint of the head with the help of a cattle halter. An injectable sedative (xylazine 0.025–0.05 mg/kg i.v.) may be used, although it is often not necessary. Federal law restricts sedatives to use by or on the order of a licensed veterinarian. The author strongly recommends the use of local anesthesia to numb the cornual nerve bilaterally with the goal to mitigate the immediate pain associated with this practice. Administering 3–5 ml of 2% lidocaine using an 18- or 20-gauge 2.5-cm needle on each side should provide adequate anesthesia of the area within three to five minutes and last for up to five hours [26, 29, 31].

Furthermore, it is imperative to mention that one should step back and consider practices that include genetic selection by utilizing polled sires, as genomics and selection make this a viable option for the future with many dairy breeds.

Common livestock procedures such as dehorning/disbudding bring up a point related to the use of antiflammatory and pain control type medications. Analgesics/anesthetics were used by 28% percent of dairy operations that disbudded/dehorned heifer calves according to the most recent NAHMS survey [25].

A recent study published in 2017 [33] looked into the effects of flunixin meglumine transdermally on pain biomarkers at the time of dehorning. Although easy to use, the group concluded that transdermal flunixin meglumine given at the time of dehorning did not provide substantial analgesia based on the pain biomarkers investigated. Differently, meloxicam orally (1 mg/kg) has been shown to

decrease pain for up to 24 hours after a single dose of the drug [33]; however, it is vital to note that there are currently no approved drugs in the USA for use in cattle with an indication to provide pain control. The Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA) allows extra-label drug use provided a valid veterinarian–client–patient relationship exists, and the drug selection process, records and withholding times outlined in the AMDUCA and current research must be followed.

According to the USDA survey in 2014, 56% of deaths of preweaned replacement heifers were due to diarrhea or other digestive disease and 24% due to respiratory disease [25]. In contrast, after weaning, respiratory disease is responsible for almost 59% of deaths. Deaths of young calves due to respiratory disease usually occur around 12 weeks of age when replacement heifers are moved from hutches into a group pen. Strict colostrum management and vaccination protocols are known to be the top key factors that must be followed in order to minimize high mortality and morbidity at this young age.

It is imperative to recognize that the use of drugs and/or nutraceuticals do not replace a competent immune system. Colostrum management, good nutrition, and solid husbandry practices are often what are needed to raise healthy calves during the first weeks of life. Heller and Chigerwe published a review that summarizes the treatment and diagnosis of the most common infectious causes of diarrhea in the neonate bovine [34]. A herd investigation of diarrheic calves is primarily accomplished by understanding the age of onset, mortality, and morbidity, as well as the prevalence and incidence of the problem [34, 35]. The age of onset is crucial to rule out the likely etiology. Table 73.2 reviews the age and etiology of calf diarrhea. However, it should be noted that the majority of enteric diseases in calves are caused by mixed infections [36], and definitive diagnosis is valuable when the incidence of diarrhea is increased within a population with the goal of addressing potential medical, biosecurity (zoonosis), husbandry, and dietary problems, whereas diagnosing individual sporadic cases is known to be of little value. Frequently, clinical signs and age of the neonate dictate the likely cause of the diarrhea and supportive care is usually the treatment option, as the majority of etiologies have no specific therapy. As suggested in a review by Constable, the main goal when treating an individual is to prevent septicemia or bacteremia, decrease the bacterial load (mainly *Escherichia coli*) in the small intestine and abomasum, prevent negative energy balance, and reduce stress by providing analgesia [37].

In general, utilizing a combination of Gram-negative spectrum antimicrobials and a non-steroidal anti-inflammatory drug, while decreasing the volume and increasing the frequency of the milk diet, are the current recommendations [37, 38]. There is a plethora of antimicrobials available to treat bacterial enteritis in diarrheic calves [38], so it is important to consider the prudent use of these drugs when it comes to following the manufacturer's label; AMDUCA guidelines by discretionary use of extra-label drugs and, most importantly, a protocol should be in place that addresses the appropriate dosing, route, and frequency that reaches and maintains an effective therapeutic concentration at the site of infection [38].

Oral electrolytes are considered the hallmark of routine treatment to achieve rehydration. Smith [38] states that these products must supply a sufficient amount of sodium to normalize extracellular fluid, provide agents that facilitate the absorption of sodium and water from the intestines into the circulatory system, correct the metabolic acidosis, and provide energy. It is extremely important to understand that oral electrolytes do not replace the milk diet and vice versa; milk must not be mixed with the oral electrolytes, nor should it be fed concomitantly. The producer must always follow label directions when it comes to mixing the electrolyte solution, as improper mixing will interfere with the osmolality of the fluid in question and diarrhea, and further severe metabolic imbalance may occur.

Table 73.2 The most common infectious causes of calf diarrhea and age of occurrence.

Etiology	Age of onset
Clostridium perfringens type C	<2 days old
Clostridium perfringens (others)	Varies with type, typically secondary
Enterotoxigenic *E. coli* (ETEC)	0–7 days old
Enterohemorragic *E. coli* (EHEC)	2–30 days old
Rotavirus	5–14 days old
Coronavirus	5–30 days old
Cryptosporidium parvum	5–30 days old
Salmonella	>5–14 days old and anytime
Nutritional	Until weaned
Giardia	2 weeks to 2 months, typically secondary
Eimeria sp.	>25 days old
Parasites (HOTC complex)	After 3 weeks old
BVDV	PI3 calves – anytime

HOTC, *Haemonchus contortus*, *Ostertagia ostertegi*, *Trichostrongyles axei*, and *Cooperia* sp; BVDV, bovine viral diarrhea virus; PI3, parainfluenza 3.

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Critical Care Management of the Neonate

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Introduction

Neonatal calf morbidity and mortality are a major economic concern for beef and dairy cattle producers worldwide. Perinatal calf mortality in the first 48 hours of life has been estimated to be between 3 and 10.3% [1, 2]. A recent study determined that 6% (greater than 500 000 calves) of the total dairy calf mortality per year in the USA occurs at birth or shortly after [2]. Previous reports indicate that between 69 and 75% of perinatal beef and dairy calf mortality in the USA occur within the first 96 hours of life [3]. Therefore it is imperative to provide immediate evaluation and care of newborn calves to ensure calf survival and health and prevent economic losses associated with perinatal mortality. Several factors including dystocia, a fetus derived from cloning or *in vitro* fertilization, prematurity, dysmaturity, congenital defects, and neonatal infection (septicemia) represent risk for calf mortality during the perinatal period and should be addressed accordingly to prevent losses.

Pathophysiology of Extrauterine Neonatal Adaptation

Physiological changes in the cardiovascular and respiratory systems of the fetus are critical for normal adaptation of the calf to the extrauterine environment and survival. During stage 2 labor, the rupture of fetal membranes and umbilical cord initiates hypoxia in the calf [4]. Reduction of oxygen and accumulation of carbon dioxide in the calf's blood induce gasping reflexes, initiation of breathing, and inflation of the lungs [5]. Reduced alveolar pressure by the action of surfactant production allows air to remain in the lungs after initiation of regular breathing [6]. As oxygenation improves, pulmonary blood flow pressure decreases and peripheral vascular resistance increases, leading to the closure of the foramen ovale and ductus arteriosus, ceasing fetal circulation [5]. The normal progression of the fetal adaptation process can be altered by dystocia or other complications during calving such as meconium aspiration, prematurity, dysmaturity, and the presence of congenital abnormalities. Metabolic acidosis in the newborn calf due to hypoxemia and hypercapnia is a common consequence of dystocia and other complications during calving [7]. Persistent hypoxemia can lead to neonatal asphyxia, hypoxic encephalopathy, persistent foramen ovale and ductus arteriosus (right to left shunting), failure of adaptation, reversion to fetal circulation, and, ultimately, death.

Initial Assessment and Management of the Newborn Calf

Physiological adaptation needs to occur in the fetus to ensure viability of the calf after birth. Calves born after gestation of normal/physiological duration and by normal (unassisted) parturition usually have minimal problems adapting to the extrauterine environment. However, calves born by dystocia or assisted parturition carry a higher risk of adaptation failure and have an increased risk of morbidity and mortality [7–9]. Common disorders observed in compromised newborn calves are hypoxemia, respiratory and metabolic acidosis, hypothermia, and failure in the transfer of passive immunity [10, 11]. Early and vigilant monitoring should be performed in calves born following dystocia or assisted calving. Under normal circumstances, calves should attain sternal recumbency during the first 2–3 minutes and should attempt to stand within 15 minutes of birth. Hypoxic and acidotic neonates

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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usually have difficulty maintaining sternal recumbency, have a weak or absent suckle reflex, and require more time to stand [12]. Results from one study demonstrated a probability of 84% for mortality in calves that took greater than 15 minutes to achieve sternal recumbency [13]. Other clinical signs, such as failure to initiate respiration, tachypnea (<10 rpm), bradycardia (<40 bpm), pale or cyanotic mucous membranes, and the presence of a swollen tongue strongly suggest hypoxemia or inadequate neonatal adaptation and need to be addressed immediately [8, 14]. Recent studies evaluating newborn beef calves reported a significant association between abnormal mucous membrane color (white, red, or blue), inability to completely withdraw the tongue when pinched, and weak suckle reflex with decreased blood pH, increased blood lactate, and failure in the transfer of passive immunity [8, 14]. In human infants, a scoring system (Apgar score) that is based on five criteria (Appearance, Pulse, Grimace, Activity, and Respiration) is used to quickly assess neonatal well-being. While modifications of the Apgar scoring system have been used to identify compromised calves early after birth, inconsistency and lack of practicality has reduced their use among veterinarians and producers [14]. Critical points in the initial management of hypoxic/acidotic calves include establishing a patent airway, stimulating respiration, maintaining adequate body temperature, and ensuring adequate transfer of passive immunity [8, 15].

Establishing a Patent Airway

The calf is placed in sternal recumbency to favor oxygen and carbon dioxide exchange. Fluid removal from the upper respiratory tract by hand or suction is important when promoting airway patency. Suction can be used to remove fluid from the upper respiratory tract; however, it should be used with caution. Suction time should not exceed 5–10-seconds per bout in order to prevent reflex vagal bradycardia [16]. Alternatively, manual suction with a bulb syringe can be used to remove excess fluid. In the opinion of the authors, newborn calves should never be suspended by the hind limbs in an attempt to clear fluid from the respiratory tract. This action can interfere with normal respiratory adaptation by increasing thoracic (pulmonary) pressure because of the abdominal viscera pressing on the diaphragm [15]. (Editor's note: Brief suspension, for a duration of less than 90 seconds, has been shown to be beneficial following delivery by C-section specifically due to a positive functional impact on postnatal pulmonary mechanics and gas exchange.) Signs of inability of the calf to start spontaneous respiration should prompt stimulatory action.

Stimulation of Respiration

Spontaneous respiration should occur within 30 seconds of birth. Assistance should be provided if the calf fails to start breathing on its own during the first one to three minutes after birth. Several non-invasive methods to stimulate respiration have been described in newborn calves including vigorous towel-rubbing of the thorax and limbs, placing a finger or a piece of straw in the calf's nostrils, and pouring cold water over the calf's head and ears [5, 17, 18]. Results from one study demonstrated that newborn calves that had 5 l of cold water (5° C, 40° F) poured on their heads immediately after birth had increased respiratory rates, increased tidal volumes, and improved pulmonary function compared with control calves with no water poured on the head [15]. The authors of another study suggested that the insertion of a 20-gauge 1-inch needle in the nasal planum could stimulate the phrenic nerve and respiration [19]. If the calf does not respond to non-invasive stimulatory methods, pharmacological stimulants and/or endotracheal intubation and mechanical positive pressure ventilation (PPV) are necessary to preserve the calf's life. In this case, PPV is aimed at initiating respiration instead of treating hypoxia [20]. Specific treatment of persistent hypoxia will be discussed in the section "Specific Perinatal Conditions of Newborn Calves".

Treatment with pharmacological respiratory stimulants such as caffeine, aminophylline, or doxapram hydrochloride has been advocated for compromised neonates and can decrease the need of PPV in field situations [10, 11]. These drugs directly stimulate the respiratory center in the medulla and can improve diaphragmatic contractility. Caffeine and aminophylline dosages are mostly adopted from human and equine medicine [11]. In contrast, results from some studies have demonstrated beneficial effects of doxapram on respiratory function and survivability of ruminant neonates [21–23]. Caffeine can be administered orally or per rectum at a loading dose of 10 mg/kg and continued with 2.5–3 mg/kg SID (once daily) as maintenance dose. Aminophylline can be used at 4–10 mg/kg intravenously (IV) BID (twice daily) or TID (three times daily) or as a CRI (constant rate infusion) at 10–30 mg/kg/day. Doxapram hydrochloride can be used at 0.5 mg/kg IV SID or, in cases of emergency resuscitation and anoxia, at 5–10 mg/kg injected at the base of the tongue [11]. Reports on the effectiveness of these medications to stimulate and improve respiratory function in neonates are varied and inconclusive. Additional controlled clinical trials are needed to make judicious recommendations on the use of respiratory stimulants in compromised neonatal calves [10].

If mechanical ventilation is needed, a 6- to 10-mm endotracheal tube is usually effective. If a rebreathing bag is going to



Figure 74.1 One-day-old calf with hypoxemia and hypercapnia.

be used, it should be equipped with a safety valve to prevent excessively high pressures and alveolar damage. The ventilation rate should be around 15–25 respirations per minute. If 100% oxygen supply is available, a flow rate of 150–200 l/min with a 50-psi line and a maximum pressure of 39 mmHg through the endotracheal tube is recommended [5, 20]. The endotracheal tube should be removed once the calf starts fighting it. When mechanical ventilation with oxygen is unavailable, intranasal humidified oxygen should be administered in combination with pharmacologic respiratory stimulants. Intranasal oxygen can be administered at a rate of 2–10 l/min through soft intranasal cannulas inserted into nasal passages and advanced at the level of the medial canthus. The exterior portion of the cannulas is sutured or stapled to the skin (Figure 74.1). Red rubber catheters (10–12 mm in diameter) can be used as soft intranasal cannulas for humidified oxygen administration in calves [6].

Cardiac Stimulation

While cardiac resuscitation is unrewarding in calves born without a heartbeat, it should be attempted immediately in bradycardic newborn calves [heart rate (HR) < 40 bpm] or those that develop asystole. Cardiac stimulation should be performed in conjunction with respiratory stimulation because severely bradycardic or asystolic calves are usually not breathing on their own. The chances of successful revival are much greater when cardiac stimulation starts before nonperfusing rhythm or asystole [10]. Epinephrine is the drug of choice for cardiac stimulation in calves. Epinephrine can be used at doses that range from 0.1–0.2 ml/kg of the 1:10000 concentration to 1 ml/45 kg of the 1:1000 concentration. It can be given IV or intratracheally every three to five minutes [24]. Cardiac compressions to assist circulation can be initiated at the same time. A cardiac compression rate of 100 per minute with 8–10 respirations per minute has been described as reasonable for newborn calves [10]. If response to epinephrine is poor and severe bradycardia or asystole continue, vasopressin at 0.4 IU/kg IV can be administered. Vasopressin is a peripheral vasoconstrictor that has resulted in positive outcomes when used during cardiopulmonary resuscitation in humans [25]. In general, rapid initiation of cardiac compressions (before asystole), establishing an appropriate compression rate (100 per minute), and establishing an airway (endotracheal intubation) are considered the most important steps of cardiopulmonary resuscitation of calves [10].

Maintaining Adequate Body Temperature

The thermostatic mechanisms that regulate body temperature after birth usually fail in calves born following a prolonged calving process or severe dystocia [26]. This is especially true during times of the year when environmental temperatures shift dramatically during the day. Rapid thermoregulation after birth is critical for the calf's adaptation and survival. Hypothermic calves (rectal temperature <98 °F or 36.5 °C) should be assisted with the use of heating devices such as electric heating pads, circulating water heating pads, or infrared heaters. One study demonstrated that exposure of newborn calves to an infrared heater for 24 hours immediately after birth resulted in improved rectal temperature, increased arterial hemoglobin oxygen saturation, increased tidal volume, increased dynamic lung compliance, and increased respiratory rates compared with calves not exposed to the heaters [15]. Caution and close monitoring are important when using electric heating devices (heating pads, lamps) as burning is possible. Once the rectal temperature of the calf is >98 °F (36.5 °C), the heating device should be removed.

Ensuring Adequate Transfer of Passive Immunity and Nutrition

High-quality maternal colostrum or a colostrum replacement product should be given to newborn calves during the first four hours of life to achieve adequate transfer of

passive immunity. The correlation between inadequate transfer of passive immunity and calf morbidity and mortality before weaning is well documented in the literature [27–31]. Recently, adequate transfer of passive immunity in dairy calves has been suggested as shown by immunoglobulin G (IgG) levels in serum at 24 hours of life >21 g/l [28]. In beef calves, adequate transfer of passive immunity is defined as serum IgG levels at 24 hours of life >24 g/l [27]. The total mass intake of IgG required to achieve these goals varies between 200 and 300 g. In beef cattle, 200–300 g of IgG can be found in a volume of 1.5–2 l of maternal colostrum. Under normal conditions, a beef calf is expected to nurse this amount from its dam. In contrast, in dairy cattle a volume of 4–6 l of good quality maternal colostrum (>50 g of IgG/l) is required to achieve 200–300 g of total IgG for the calf. In hypoxic newborns, smaller volumes of colostrum at greater frequencies (30 minutes–1 hour) should be fed by bottle or esophageal tube to achieve complete intake. In addition to passive immunity, maternal colostrum provides newborn calves with nutrition, vitamins, and growth factors essential for calf survival during the first hours to days of life. In cases of confirmed failure of transfer of passive immunity in calves older than one day, fresh or frozen plasma (30 ml/kg IV) from their dam or a healthy donor from the same farm should be given. After colostrum feeding, nursing from the dam should be encouraged and monitored in beef calves. Alternatively, pasteurized whole milk or milk replacer at 12–20% of body weight per day should be fed at smaller volumes every three to four hours. In calves with poor or absent suckle reflex that tolerate tube-feeding, repeated tube feedings may be necessary. In calves that do not tolerate tube feeding (e.g. bloating, regurgitation) parenteral nutrition should be considered [32].

Clinical Pathology

To evaluate respiratory function and ventilatory capabilities of newborn calves, arterial blood gas measurement is considered the gold standard. Arterial PaO_2, $PaCO_2$, bicarbonate (HCO_3) concentration, SaO_2, and blood pH values should be evaluated. Reference values for newborn calves from birth to 24 hours of life are shown in Table 74.1 [33]. Although venous blood could be used for measurement of blood pH, HCO_3 concentration, and base excess, arterial blood is required for measurement of PaO_2 and $PaCO_2$ because of the poor correlation between arterial values with venous blood values [4, 34]. The caudal auricular artery (medial intermediate branch) and the brachial artery are commonly used by practitioners to obtain arterial blood samples. After properly identifying the arteries in their respective anatomic locations, blood can be collected using either a commercially available arterial blood gas sampling kit or a heparinized tuberculin syringe and a 25-gauge needle. After sample collection, the air from the syringe should be evacuated and the needle capped with a rubber stopper until analyzed. Samples should be sealed and submitted for analysis as soon as possible; however, if sealed correctly and placed on ice, samples can provide reliable results up to four hours after collection. Blood gas analysis is usually performed routinely in referral centers, but it can also be performed in field conditions with a commercial blood gas analyzer (i-Stat Corporation, East Windsor, NJ). Newborn calves that fail to adapt to the extrauterine environment commonly develop persistent hypoxemia, hypercapnia, and combined respiratory and metabolic acidosis [11].

Pulse oximetry is another non-invasive and immediate technique that has adequate accuracy to measure oxygen saturation of hemoglobin (SaO_2) in newborn calves. However, the reliability of this technique is lesser in cases

Table 74.1 Mean and (SD) values of blood gas analyses of arterial blood in 57 neonatal calves from birth to 24 hours of age.

Variable	At birth	0.5 h	4 h	12 h	24 h	P^a	P^b
pH	7.3 (0.06)	7.36 (0.04)	7.38 (0.03)	7.42 (0.03)	7.43 (0.04)	< 0.001	>0.05
PCO_2	57.31 (4.5)	52.58 (5)	48.7 (3.7)	43.7 (4.75)	44.22 (4.3)	<0.001	<0.001
PO2	45.31 (16)	58.1 (13.1)	67.6 (14.5)	71.89 (8.3)	66.77 (14.2)	<0.001	<0.05
HCO_3	26.76 (3.4)	28.01 (2.4)	27.4 (2.32)	26.96 (2.94)	28.31 (3.26)	>0.05	>0.05
Base excess	0.86 (4.12)	2.9 (2.88)	2.52 (2.64)	2.78 (3.23)	4.42 (3.6)	>0.05	>0.05
SO_2	64.2 (20.8)	82.1 (9.98)	89.23 (6.84)	92.84 (2.32)	89.75 (8.3)	<0.001	>0.05

Source: Adapted from [33].
HCO_3, Bicarbonate; PCO_2, partial pressure carbon dioxide; PO_2, partial pressure oxygen; SO_2, oxygen saturation.
[a] Analysis of variance for repeated measures within groups.
[b] Analysis of variance for repeated measures between groups.

of low oxygen saturation (<70%), carboxihemoglobinemia, methemoglobinemia, hypotension, anemia (Hb < 3 g/dl), or dark-pigmented skin [24]. The oximeter sensor can be placed on non-pigmented skin or mucosa such as the vulva, the prepuce, or the lip. Oximetry values <90% represent desaturation and oxygenation disturbance in most species. In addition to blood gas analysis, blood glucose, complete serum chemistry profile, and a complete blood count (CBC) can provide insight on the energy and electrolyte balance, liver and renal function, and probability of neonatal infection. The presence of hypoglycemia and metabolic and electrolyte abnormalities should be corrected with appropriate IV fluid therapy. Neonatal infection carries a more guarded prognosis and management includes IV fluids, plasma, systemic antibiotics, and non-steroidal anti-inflammatory drugs [35, 36].

Specific Perinatal Conditions of Newborn Calves

Persistent Hypoxemia

Dystocia can result in severe respiratory and metabolic acidosis in the fetus due to excessive accumulation of carbon dioxide in the blood. Healthy newborn calves born following dystocia can usually self-correct hypercapnia within a few hours after birth as respiration continues and oxygenation improves [7]. However, under certain circumstances, such as prolonged dystocia, premature calf, low concentration of inspired oxygen, bacterial or viral pneumonia, and congenital respiratory or cardiovascular abnormalities, hypoxemia persists and becomes life threatening [24]. Clinical signs are observed immediately after birth and tend to worsen over time. Signs such as inability to maintain sternal recumbency, inability to stand or difficulty in standing, weak or absent suckle reflex, hypothermia (rectal temperature <98 °F or 36.5 °C), low or infrequent breathing, and bradycardia (<80 bpm) are usually observed [12]. Other clinical signs may be present depending on the initial cause of adaptation failure and should be identified quickly through a thorough physical examination. Neurological signs such as severe depression, unresponsiveness, and seizure activity suggest hypoxic encephalopathy.

Diagnosis of persistent hypoxemia is based on history, clinical signs, and arterial blood gas analysis. Hypoxemia (PaO_2 < 45 mmHg), hypercapnia ($PaCO_2$ > 57 mmHg), and acidemia (blood pH <7.3) are common abnormalities found in these calves [7, 11, 12]. Treatment of persistent hypoxemia/hypercapnia in field conditions can be challenging. Affected calves should be started on intranasal humidified oxygen at 2–10 l/min. Respiratory stimulants such as doxapram hydrochloride, aminophylline, and caffeine at the doses described in the section "Initial Assessment and Management of the Newborn Calf" can be used to improve respiratory function. Sodium bicarbonate ($NaHCO_3$) therapy to combat metabolic acidosis is not recommended in hypoxic calves with abnormal ventilation as it could intensify hypercapnia and lead to cerebral acidosis [6]. In cases of hypoxic encephalopathy, treatment with furosemide (1 mg/kg IV BID), mannitol (0–0.5–1 g/kg IV as a 20% solution), and dimethyl sulfoxide (DMSO) (0.5–1 g/kg IV as a 10% solution) could aid in the treatment of cerebral edema; however, this therapeutic regime is rarely effective [24]. Ideally, repeated blood gas measurements indicate response to therapy. Calves with poor response after 12 hours of intranasal oxygen have a poor prognosis and may require mechanical ventilation.

Persistent Pulmonary Hypertension

This syndrome occurs in neonatal calves that undergo a delayed transition from fetal pulmonary hypertension to low resistance pulmonary circulation. Vasoconstriction of pulmonary vessels in response to persistent hypoxemia and reversion to fetal circulating patterns (right to left shunting) are the major contributing factors to pulmonary hypertension. This condition has been described in human infants and foals, and more recently in cloned calves [37–39]. Factors such as failure of the neonate's cardiopulmonary circulation to transition from the fetal state, meconium aspiration, high altitude, and pneumonia have been associated with its presentation in calves [38]. Clinical signs are observed immediately after birth and are similar to those described for calves with persistent hypoxia. Diagnosis is based on history, clinical signs, blood gas analysis, diagnostic imaging, and increased pulmonary arterial pressure. Repeated arterial blood gas analyses demonstrate persistent or progressive hypoxemia (PaO_2 <80 mmHg). Thoracic radiographs and computed tomography (CT) scan usually demonstrate atelectasis with decreased vascular patterns in the lungs. Cardiac catheterization demonstrates increased pressures in the pulmonary artery, right ventricle, and right atrium [11, 24]. Treatment of this condition consists of intranasal oxygen supplementation and mechanical ventilation if hypoxemia and hypercapnia persist. In calves unresponsive to mechanical ventilation, nitric oxide at 5–40 ppm in the ventilator gas can be attempted to promote peripheral vasodilation [24]. Additionally, endothelin-1 (ET-1) receptor antagonists such as sildenafil administered orally or as a suppository have been used as alternative vasodilators in humans [40]. The prognosis of persistent pulmonary hypertension depends on the initial cause of the disease. Non-response to oxygen therapy is also associated with a poor prognosis.

Respiratory Distress Syndrome

Disorders caused by incomplete organ development are usually seen in premature calves. In the case of the lungs, this can be life threatening, as affected calves can present with progressive respiratory difficulty, tachypnea, increased inspiratory and expiratory effort, hypoxemia, and ultimately death [41]. This is known as respiratory distress syndrome (RDS) and has been associated with inadequate surfactant production in prematurely born calves [42]. The surfactant system in the lungs is completely developed in the fetus at around 270 days of gestation. Calves born before 270 days of gestation have an increased risk of developing RDS due to lack of adequate amounts of surfactant production [43]. Surfactant is essential to prevent pulmonary collapse during breathing by reducing alveolar tension and maintaining alveolar stability. Insufficient surfactant production results in end-expiratory collapse and reduced gas exchange surface in the lungs. As the need for oxygen increases, the calf's inability to maintain adequate ventilation leads to progressive hypoxemia. Aspiration of meconium also impairs surfactant function and can promote RDS. Meconium causes an inflammatory reaction in the lungs, affecting function of type 2 pneumocytes and decreasing surfactant production [44]. Calves affected by RDS are usually premature as evidenced by the following physical characteristics: rounded heads, small body size, silky short hair, short umbilical hair, and incomplete or absent eruption of incisors. Clinical signs of progressive respiratory difficulty including tachypnea, inspiratory and expiratory dyspnea, apnea, severe lethargy, and pale or cyanotic mucous membranes are commonly observed 15–60 minutes after birth in calves with RDS [41].

Diagnosis of RDS is based on history, clinical signs, and the presence of severe hypoxemia (PaO_2 <45 mmHg), hypercapnia ($PaCO_2$ >58 mmHg), and acidemia (blood pH <7.2). In one study, the mean PaO_2, $PaCO_2$, and blood pH of 20 premature calves with RDS was 29.6 mmHg, 67.7 mmHg, and 7.1, respectively [41]. Radiographs of the lungs 24–48 hours after birth can aid in the diagnosis of RDS by demonstrating the degree of atelectasis, lung volume, and lung density in affected calves [38]. Treatment of calves with RDS consists of intranasal oxygen and mechanical ventilation in cases of persistent hypoxemia and hypercapnia. Results from one study demonstrated an increase of PaO_2 from 38.4 to 58.7 mmHg in calves with RDS within three hours of intranasal oxygen therapy (5–6 l/min) with no additional treatments [45]. Crude bovine surfactant collected from freshly slaughtered healthy cattle has been advocated in the treatment of RDS in calves. Intratracheal administration (via endotracheal tube) of a single dose of bovine surfactant (100 mg/kg) to calves with RDS resulted in a reduction of mortality by 60% in the treatment group compared with the control group [41]. Unfortunately, the cost of surfactant therapy at the reported dosage and with commercially available surfactant products is cost prohibitive for most producers (USD $12 000–16 000 for a single dose). Glucocorticosteroid therapy is a common and widespread practice for treatment of RDS among producers and veterinarians. Dexamethasone treatment appears to improve and favor lung maturation and surfactant production in the newborn calf [42, 46]; however, evidence of the efficacy of dexamethasone in the treatment of RDS in calves is lacking in the literature.

Prevention of Perinatal Mortality

Prevention of dystocia and reduction in the risk of premature calving should be the basis for prevention of perinatal mortality. All calves born before 270 days of gestation have an increased risk of developing RDS. Therefore avoidance of stressful management situations that can result in premature calving is a key factor in prevention. After 270 days of gestation, pregnant cows should not be transported, hoof trimmed (tilt table/chute), sedated with α2 agonists, or exposed to heat for long periods. High-risk pregnancies (cloned calves) or pregnancies carrying embryos of high genetic value need to be monitored through ultrasonography during the last trimester of gestation (every two weeks) for evaluation of the fetomaternal junction and early detection of edema hydrallantois, or other problems [47]. These findings decrease fetal viability, and decisions on induction of parturition or elective cesarean section should be made. A combination of dexamethasone and dinoprost (PGF2α) to induce parturition in cattle after 260 days of gestation reduces the risk of RDS in calves by accelerating type 2 pneumocyte maturation and surfactant production [42, 46]. Induction of parturition in cows under 260 days of gestation (230–260 days) to prevent RDS in the calf is possible with a modified seven-day induction regime [48]. Dexamethasone (5 mg) is given intramuscularly (IM) BID for four days. On day 5, 10 mg of dexamethasone is given IM BID. On day 6, 40 mg of dexamethasone and 25 mg of dinoprost are given IM once. Parturition usually occurs after day 6 [48].

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Colostrum: A Review

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Introduction

Maternal colostrum contains immunological and nutritional components that are essential for the calf's survival and health during the first months of life. Immunoglobulins (Ig) and leukocytes present in colostrum provide the newborn with specific immunity against common infectious pathogens. Additionally, nutritional components of colostrum such as complex carbohydrates, fat, protein, and minerals provide energy and modulate activation of cell metabolism to guarantee the calf's survival during the first 24 hours of life. The association between overall dairy- and beef-calf health and adequate colostrum intake has been previously documented in the literature [1–4]. Adequate colostrum intake is related to the ingestion of an acceptable quantity of high quality colostrum in a timely manner and usually results in adequate transfer of passive immunity (API) to the calf. In contrast, a lack of adequate colostrum intake usually results in failure in the transfer of passive immunity (FTPI). FTPI is a condition associated with inadequate levels of colostrum derived Ig and other immune factors in the calf's serum at 24 hours of age. Calves that develop FTPI have greater morbidity and mortality rates before weaning compared with calves with API [4–6]. To prevent FTPI, newborn calves should receive an adequate amount of high quality maternal colostrum or a commercial colostrum replacement product within the first six hours of life. Monitoring the transfer of passive immunity in newborn calves at the individual-calf and herd levels is a key management strategy to reduce economic losses associated with calf morbidity and mortality. Evaluation of the level of absorption of passive immunity is usually performed through several on-farm and laboratory tests. This allows producers and veterinarians to make corrective decisions on overall colostrum management.

The Cow

Placenta

Placentation in the ruminant is described as synepitheliochorial and thus in utero passage of Ig from the dam to the fetus is prevented. This type of placentation forms a syncytium between the maternal endometrium and the fetal trophectoderm, separating the maternal and fetal blood supplies [7]. As a result, calves are born agammaglobulinemic and dependent on the absorption of Ig, leukocytes, and other immune factors present in maternal colostrum to achieve API.

Colostrogenesis and Components of Colostrum

Colostrum contains not only Ig but also other factors that help with establishment of the calf's immune system and prevention of disease. Colostrogenesis occurs several weeks prior to parturition and ceases abruptly immediately prior to parturition [8]. Physiologic changes, specifically endocrine control, coincide with the absorption of Ig across the mammary epithelium. Endocrine changes that have been shown in the weeks leading up to parturition include increased estrogen levels; increased serum corticosteroid, growth hormone, and prolactin; and a severe decrease in progesterone. Previous research has demonstrated that estrogen, alone or with progesterone, initiates the IgG1 Fc-receptor of the neonate (FcRn) within the mammary gland [8, 9]. These receptors are specific to the Fc portion of Ig [10]. The FcRn contains subunits and the expression of these, along with intracellular proteins, is upregulated by both estrogen and progesterone, which in turn increases transport of Ig across the mammary epithelium. Transport and release of Ig across the mammary epithelium and into the alveolar lumen occurs by transcytosis [7, 10]. Bovine colostrum is a mixture of lacteal derived secretions, proteins, leukocytes, growth factors, hormones,

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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vitamins, minerals, non-specific antimicrobial factors (lactoferrin), and other nutrients. Microelements, such as zinc and selenium, are shown to have increased concentrations in bovine colostrum [11]. Variability of the components of colostrum is likely but not limited to factors such as nutrition, breed, season, and age [12]. A review recently published expands on these effects in dairy cattle [13].

Absorption of Ig is crucial for the prevention of FTPI and disease. The predominant Ig found in bovine colostrum is IgG, specifically IgG1, accounting for 75–90% of the total IgG. IgA and IgM account for approximately 5 and 7% of the total Ig in colostrum, respectively. While IgG is transported through the alveolar epithelium by a receptor-mediated mechanism, IgM and IgA are locally produced in the mammary gland by plasmacytes [13, 14]. Varying concentrations of Ig are found in subsequent lactations and also when comparing cows to heifers [15]. The role of Ig in colostrum was described in 1969 [16]. IgG1 from maternal colostrum is the most important component of passive immunity in the calf. IgG1 is the only Ig capable of being resecreted to the enteric and respiratory tract mucosa to provide specific immunity and protection against common infectious diseases. IgG antibodies can activate complement and sensitize bovine skin [16]. IgM is important in opsonization, neutralization of viruses, and complement activation [17, 18]. IgA is found in the majority of mucosal secretions and prevents infection by agglutination of infectious agents. Additionally, IgA contributes to colonization of commensal bacteria within the gut, respiratory tract, and ocular mucosa [10, 18–20].

Another important component of colostrum is maternal leukocytes. The cell populations present in descending order include macrophages, neutrophils, and lymphocytes. Research has demonstrated that maternal leukocytes from colostrum enhance the development of the neonatal immune system [21]. The leukocytes that are present in colostrum and then absorbed into neonatal circulation are immunocompetent and can facilitate antibody production by enhancing activation of the calf's naïve T lymphocytes [22, 23]. The process of heat treatment, freezing, and thawing results in depletion of maternal leukocytes from colostrum [13, 24]. The role of maternal leukocytes present in colostrum on cell-mediated immune responses of the calf has been studied [13, 22, 25–29]. Results from one study demonstrated early (one week) cell-mediated immune responses in calves fed whole colostrum compared with late (two to three weeks) cell-mediated immune responses observed in calves fed acellular colostrum [28]. Another study evaluated neonatal calves that were fed fresh, frozen, or acellular colostrum, and their peripheral blood mononuclear cells (PBMC) response against bovine viral diarrhea virus (BVDV) and mycobacterial protein derivatives was assessed [27]. In this study, the cows from which the colostrum was harvested had all been vaccinated against BVDV. At 24 hours of life, PBMC responses were detected only in calves receiving fresh colostrum, not in calves receiving frozen or acellular colostrum. Similarly, another study evaluated fecal shedding of *Escherichia coli* after calves were fed whole colostrum versus acellular colostrum. The calves were inoculated with *E. coli* three hours after birth and then fed colostrum treatments. Calves that received whole colostrum shed less *E. coli* in their feces and had higher levels of both IgA and IgM [26]. Immune responses to naturally occurring respiratory and enteric disease were not different in calves fed cellular vs acellular colostrum [13, 30, 31].

Other components of colostrum that are essential for the calf in early life include proteins different from Ig, fat, cytokines, nutrients, antimicrobial factors, hormones, and growth factors. As stated by McGrath et al., "in general, colostrum contains less lactose and more fat, protein, peptides, non-protein nitrogen, ash, vitamins and minerals, hormones, growth factors, cytokines, and nucleotides than mature milk" [32]. Many of these factors are essential for the development of the gastrointestinal tract, protection of the Ig, endogenous glucose production, and thermoregulation [13, 32–34]. One example of these factors is trypsin inhibitor enzymes, which protect the Ig from proteolytic degradation in the calf's intestine [13, 32]. The energy that colostrum supplies as the first milk is essential for thermoregulation and calf survival during the first 24 hours of life, especially for calves born in the winter and during extreme cold conditions [35]. The major growth factors in colostrum include insulin-like growth factor (IGF) and transforming growth factor beta-2 (TGFβ-2) [33, 36]. IGF is essential for early intestinal transport, metabolism of glucose, synthesis of proteins, DNA, RNA, regulation of cell proliferation, and inhibition of apoptosis. Also, IGF has been shown to increase the villus size of enteric cells which allows for increased absorption of nutrients within the intestine [13, 36–38]. Other components of colostrum are vitamins. Colostrum intake is important for normal plasma concentrations of β-carotene, vitamin A, and vitamin E. Lack of adequate vitamin levels due to delayed colostrum intake (>12 hours) can result in decreased fatty acid synthesis and failure of the cell membrane protein pumps. This does not hold true for water-soluble vitamins. Many of these aforementioned components have been and still are being studied to understand how important they are in the protection of the calf and API.

Colostrum Intake

Beef and dairy calves are not the same in regards to how they are raised and how colostrum is provided. The most recent National Animal Health Monitoring System (NAHMS) dairy

study (2014) indicated that on dairy farms with greater than 30 cows, 95.4% of owners hand-fed colostrum to at least some calves [39]. On the very small operations (<30 cows), 16.8% of calves suckled from the dam [39]. Overall, 53.2% of dairy calves were hand-fed colostrum. On the beef-calf side, usually the calf naturally suckles the dam and therefore there are still a lot of uncertainties because consumption and assessment of colostrum are difficult to quantify. One survey of beef cow-calf producers in the USA demonstrated that 86.1% of producers intervened with colostrum consumption because they subjectively deemed the calf did not suckle enough [40]. The type of colostrum administered varied with each calf but the majority was from the calf's dam [40]. Studies evaluating beef cow colostrum and appropriate quantities needed are lacking.

Absorption of large proteins across the intestinal wall is a very non-selective process. During the first 24 hours, Ig and other molecules are absorbed through the small intestine by endocytosis. The large molecules are transported to the intestinal cell membrane where they are exocytosed into the lymphatics or portal circulation and thus gain entry into the systemic circulation [7, 13, 41]. The non-selectivity of Ig absorption has been proven with elevated concentrations of other proteins and enzymes, such as γ-glutamyl transferase (GGT) and even bacteria such as *E. coli*. Bacterial contamination of colostrum is common during suboptimal hygienic conditions of colostrum harvesting and can result in exposure of the calf to infectious pathogens during colostrum feeding. Colonization of the intestine and absorption of *E. coli* can result in neonatal infection, including septicemia and enteric disease [41, 42]. One explanation for this is that bacteria compete with the same sites that Ig will use for uptake across the intestinal cells. It is also possible that disruption of microvilli induced by bacteria prevents absorption of other large molecules including Ig [41]. Although bacterial contamination of colostrum is more common on dairy farms where colostrum is artificially fed to calves, beef calves are just as susceptible to enteric disease in the early neonatal period if colostrum contamination with environmental pathogens occurs.

Intestinal absorption of Ig from colostrum ends when activation of lysosomes within enterocytes starts to break down protein, including Ig. This process is commonly known as "gut closure." The ability of the intestine in the neonatal calf to absorb Ig past 24 hours is minimal. Ig concentration in colostrum begins to decline once parturition has occurred. This is of particular importance in the dairy cow in relation to milking. Studies have shown that there is a decrease in the Ig concentration of colostrum over time and with subsequent milkings in dairy cattle [43–45]. One study showed that for every hour post calving there was a 3.7% decrease in colostral IgG [13, 46]. Certainly, this concept would also be relative to the beef cow and the importance of the calf suckling within the first hours after birth. Apparent efficiency of absorption (percentage AEA) of colostral Ig by the intestine has a sustained increase within the first six hours, followed by a steady decline. Ideally, the IgG AEA from colostrum should be between 30 and 35% (Figure 75.1). In contrast, serum Ig in the calf demonstrates a steady increase after six hours of life following adequate colostrum intake [7, 47, 52]. Results from one study demonstrated that in Holstein male calves, absorption of Ig similarly decreased approximately 16% at 6 and 12 hours of life [47]. Goals for colostrum intake, to avoid FTPI, and for maximum absorption should be to supply the calf with adequate, good quality colostrum within the first six hours after birth [7, 13, 52, 53].

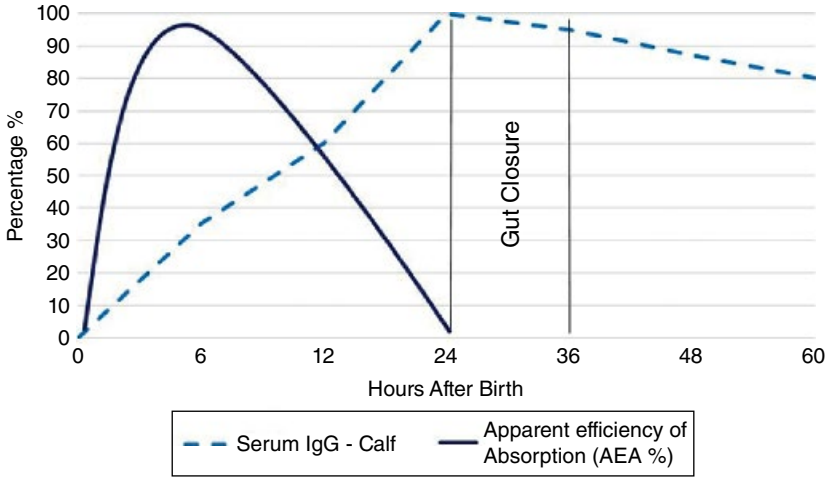


Figure 75.1 Relationship between colostrum absorption and serum Ig of the calf. *Sources:* [3, 47–51, 66].

Colostral intake, time after birth, and Ig concentration of colostrum are important to reach API in calves. Results from studies in beef and dairy calves have demonstrated that a lower serum IgG level at 24 hours of life as a result of inadequate colostrum intake is the "most important factor affecting pre-weaning health and survival" [2, 4, 6, 52, 54, 55].

Many of the guidelines for colostrum administration and feeding are based on dairy studies. This concept is not surprising, simply because of how dairy animals are raised and the ease of sampling; however, beef calf colostrum intake, administration, and supplementation are increasingly being studied. Recommendations throughout time have been that dairy calves should receive approximately 10% of their body weight (BW) in colostrum [12, 56]. One study evaluating different volumes of colostrum based on BW (7, 8.5, 10% BW) found that dairy calves fed at 8.5% BW had greater serum IgG concentrations than the other two percentages within the first two days of life [57]. The amount of colostrum supplemented to beef calves that failed to suckle adequately is normally between 0.5 and 1.25 l in most studies or a volume equal to 10% of the calf's BW [3, 52, 58, 59]. It has been shown that beef calves fed 5% of their BW within one hour of birth, followed by either suckling the dam or being tube-fed six to eight hours after, had API [60]. Differing amounts of colostrum needed are known to be due to the concentration of IgG in colostrum [61]. Beef cows have a higher grams per liter Ig concentration than dairy cows [62, 63]. Likely this is due to the volume of colostrum produced at the first milking, genetic factors, and management [12, 63, 64].A study showed that when comparing dairy to beef cow colostrum, beef cow colostrum was two times more concentrated with IgG than was dairy (65.5 g/l for dairy vs 143.2 g/l for beef) [63]. Other studies have found on average IgG concentrations of 95.9 g/l in beef cow colostrum and 64–65 g/l in dairy cow colostrum [61, 63, 65]. Further, when comparing beef and dairy calves in regards to colostrum supplementation, size of calf, quality of colostrum, and time of feeding are very important.

Colostral Volume and Time of Consumption

Consumption of colostrum can occur naturally by suckling directly from the dam or through artificial human intervention with a nipple bottle or an esophageal feeder. As a general rule that can be extrapolated from current literature, the average dairy and beef calf should receive between 280 and 300 g of total IgG mass from colostrum during the first six hours of life [6]. In dairy operations, this could be achieved with the administration of 4–6 l of good quality maternal colostrum (>50 g IgG/l). In beef calves, this could be achieved by suckling 2–3 l of maternal colostrum. Results from a study demonstrated that the mean colostral IgG concentrations in Holsteins was 74.4 g/l and over three-quarters of the samples had >50 g/l. [66] Therefore feeding protocols in colostrum management programs on dairies should be based on assessing colostrum quality and measuring colostrum IgG concentrations.

Removal of the dairy calf from the dam after birth is imperative. The dairy calf does not consistently suckle the appropriate quantity of colostrum required, and there is increased risk of exposure to pathogens when left with the dam [13]. Administration of colostrum to the dairy calf should be achieved with either a nipple system or use of an esophageal tube-feeding device. Early studies questioned the efficacy of tube feeding and IgG absorption once administered [67]. Tube feeding resulted in adequate API in those calves fed sufficient volumes of colostrum (3 l or greater). One of the main concerns with feeding colostrum using an esophageal tube is that the fluid will remain in the reticulorumen for an extended period, negatively affecting absorption of IgG (AEA). Outflow of colostrum from the rumen to the abomasum and small intestine takes approximately three hours, so as long as sufficient quantity of good quality colostrum is administered, API should be achieved [13, 68]. There was also no difference in serum IgG levels in calves fed the bottle versus tube feeding in this study [68]. A proceedings abstract evaluated beef calves suckling from the dam, bottle feeding, and two different esophageal tube-feeding times (immediately or four hours after birth). The group found that calves suckling from the dam achieved the highest IgG concentration; however, the levels did not differ significantly in those calves fed with the tube [69]. Beef calves (unassisted delivery) should be standing and suckling within one and four hours after birth, respectively [52, 59]. Results from one study demonstrated that beef calves not consuming colostrum within four hours of birth were 6.4 times less likely to reach API [52]. Colostral supplementation is likely required in the beef calf that experiences a prolonged or assisted delivery. In a study evaluating subclinical trauma during calving and transfer of passive immunity, beef calves experiencing a difficult delivery or a C-section had greater risk of FTPI [58]. If supplementation of a beef calf is required, placing the dam in a chute and allowing the calf to suckle for approximately 15–20 minutes is preferred. If the calf does not have an adequate suckle reflex, the cow should be milked and colostrum provided to the calf. Results from studies evaluating suckle reflex responses in beef calves demonstrated that calves with poor or weak suckle reflex responses had 41.6 times greater odds of failing to consume colostrum within four hours and developing FTPI [52, 58].

Colostrum Source

Sources of colostrum include the following: maternal (pooled versus non-pooled), colostrum replacers (CRs) (lacteal, serum, plasma, or whey) [70], and colostrum supplements. Maternally derived colostrum is considered the gold standard for both dairy and beef calves [7, 13]. As already stated, beef calves are expected to suckle from the dam and if there are any complications (dystocia or delayed suckle response) then intervention should occur and colostrum must be provided to reach API. Beef producers should keep a stockpile of colostrum products, including frozen colostrum from cows on the farm, high quality dairy cow colostrum, or ideally a colostrum-based CR [71]. While beef cows are not considered to make a large volume of colostrum, when possible collection and freezing of colostrum should be performed on the farm. Beef cow colostrum could be collected from cases of dystocia with a dead fetus or from high-producing beef cows. Maternal colostrum collected immediately after calving contains the highest IgG concentration. As time passes and collection of colostrum is delayed, other mammary gland secretions dilute IgG and colostrum quality drops. Results from one study demonstrated that delayed colostrum collection by 6, 10, and 14 hours after calving resulted in a reduction of colostrum IgG concentrations by 17, 27, and 33%, respectively [45]. Once collected, fresh colostrum should be refrigerated or frozen within one hour of collection. Refrigerated colostrum can be used up to 12 hours after collection but not more. Frozen colostrum stored at −20 °C can be stored and used up until one year and still be effective. Proper collection and storage of maternal colostrum is a key management strategy to prevent bacterial contamination and FTPI.

Dairy farms usually have access to a large amount of colostrum and should be able to utilize it appropriately. Pooled versus non-pooled colostrum has been evaluated extensively within the dairy industry [48, 53, 72, 73]. Pooled colostrum has mainly been used to improve the quality of fair quality colostrum and potentially maintain an adequate supply in times of enhanced need [53]. Additionally, pooling of colostrum increases the risk of transmission of colostrum-borne pathogens such as *Mycobacterium avium* subsp. *paratuberculosis*, *Mycoplasma bovis*, *Salmonella* spp., and bovine leukemia virus (BLV) to newborn calves [12, 53, 74–78]. One report indicated that the use of pooled colostrum still occurs on all-sized dairy farms but is more common on medium (100–499 cows) and large (>500) operations compared to small farms (30–99) [39]. The same report indicated that 55.1% of heifer calves on all operations were fed unpasteurized individual-cow colostrum and the remainder were either fed pooled colostrum, a CR, or other product [39]. Results from another study demonstrated that 70% of calves fed pooled maternal colostrum developed FTPI compared with only 11% of calves fed a CR product [79]. Another report indicated that when feeding calves single-cow maternal colostrum compared to a CR, the proportion of calves with FTPI was 28 and 93%, respectively [80, 81]. Feeding pooled and non-pooled maternal colostrum (dam or different dam) to dairy calves resulted in API in all calves, and no significant differences in colostral IgG were demonstrated in another report [82]. Bacterial contamination of maternal colostrum reduces absorption of IgG in the intestine and increases the risk of FTPI and neonatal infection. One study demonstrated that feeding calves with colostrum containing high bacterial counts (>1 000 000 colony forming units [cfu]/ml) resulted in reduced efficiency of IgG absorption and lower serum IgG at 24 hours after birth [83, 84]. Properly harvested maternal colostrum (beef or dairy) should contain <100 000 cfu/ml [13, 56]. The intensity of colostrum management (testing, collection, pasteurization, storage) and colostrum pooling strategies can influence success or failure in the prevention of FTPI in dairy calves; however, colostrum pooling strategies have not been studied in beef cattle.

An alternative source of colostrum when high quality maternal colostrum is unavailable or prevention of colostrum-borne pathogens is warranted on the farm is the use of commercial CR or colostrum supplement products. Other reasons such as convenience and consistency can make these products appealing to producers and veterinarians [13, 85]. CRs can be utilized on dairy farms that do not collect, store, or feed maternal colostrum, or that have abnormal health events at calving, or when cows leak colostrum before collection [86]. Replacers can also be utilized in the face of a maternal colostrum shortage. This could occur in times of calving clustering, discarding colostrum from cows that test positive for transmissible diseases (Johne's disease, *Salmonella* spp., *M. bovis*, BLV, BVDV, etc.), or on farms that do not practice colostrum pasteurization and storage. CRs are designed to be a "true substitute" for maternal colostrum and are mainly colostrum or plasma derived [80]. Replacers also contain vitamins, minerals, fat, and protein that are important for the early life of the neonatal calf. Colostrum derived CRs (lacteal) are a product of fresh frozen bovine colostrum collected from Grade A dairies. The colostrum is processed at a manufacturing plant where it is pooled and undergoes heat treatment or irradiation, spray drying, IgG testing and standardization, and packaging [87, 88]. Blood, from US Department of Agriculture (USDA) inspected abattoirs, is collected to make plasma/serum derived CRs [87]. Blood is centrifuged for separation of serum/plasma and red blood cells. The

plasma fraction is spray dried and then powdered to achieve a final Ig concentration of approximately 20 and 75% crude protein [89]. Plasma derived CRs are deficient in fats, vitamins, and minerals and thus additives are supplemented to these type of colostrum products. While colostrum derived CRs contain >90% of IgG1, plasma derived CRs contain IgG1 and IgG2 in similar concentrations and distribution. The only Ig capable of being retransferred to the respiratory and enteric mucosa after colostrum absorption in the calf is IgG1. Results of one study demonstrated that the level of neutralizing BRSV-IgG1 present in nasal secretions at 24 hours of age was significantly higher in neonatal dairy calves fed a colostrum-based CR product compared with calves fed a plasma-based product [71]. This suggests that colostrum-based products not only provide additional nutritional value but also provide specific immunity where the calf needs it the most. A licensed CR product should contain ≥100 g of IgG per packet. Not all CRs in the USA are licensed by the Center for Veterinary Biologics (CVB) and thus variability of products on the market exists especially in regard to the amount of IgG provided. Colostral products licensed by the USDA CVB and with the claim "to aid in the treatment of failure of passive transfer" must go through testing to ensure efficacy, potency, and safety [13, 90]. Other countries require all colostrum products to be licensed by their respective agencies.

Colostrum supplements are *not* intended to replace maternal colostrum. Also, colostrum supplements and CRs should never be used interchangeably as they do not provide the same benefit to the calf. Colostrum supplements are designed to do what their name implies – provide a supplement to a poor quality colostrum or a low volume of colostrum. Supplements provide between 25 and 60 g of IgG per package [87]. Colostrum supplements will not provide API and should not be used as such. Studies evaluating the use of colostrum supplements have demonstrated that if fed alone, calves will develop FTPI [91]. No added benefit of using a colostrum supplement has been reported on farms where a good colostrum program is in place [13, 92]. An appeal of colostrum supplements is their price. The cost of these products is approximately 50% of that of a CR (~USD $13 vs $26, respectively).

The validity of CRs and supplements has been thoroughly studied and evaluation of API investigated in the last 30 years [53, 79, 80, 85, 91, 93, 94]. Mixed results in regards to transfer of passive immunity have been reported in these studies. These studies have compared CRs to maternal colostrum, colostrum supplements, and lacteal or serum derived replacers. Results of one study demonstrated that only calves fed 4 l of pooled maternal colostrum containing 100 g of IgG acquired API compared to calves fed 4 l of colostrum supplements of different origin and with different IgG concentrations (100, 50, 60, and 90 g of Ig in each product). The serum IgG concentrations at 24 hours were 12.1, 6.8, 3.5, and 2.2 g of IgG/l in study calves fed pooled maternal colostrum, a serum based colostrum supplement, and two different milk based colostrum supplements, respectively [94]. Another study comparing maternal colostrum, serum derived CRs, and colostrum supplements found that the total mass of IgG fed and processing method were critical to ensure API [89]. This group also found that maternal colostrum conveyed API, whereas the replacer and supplement did not (18.8 g/l vs 8.35 g/l and 6.62 g/l, respectively). The main explanation for this was the amount of IgG present in maternal colostrum. Calves that were fed a plasma derived CR exhibited lower serum IgG and total protein concentrations between one and eight days of age compared with calves fed maternal colostrum in one study. Interestingly, in this study although there was a trend, the number of calves treated for illness was not significantly different between the two groups [80]. Serum IgG at 24 hours of age was significantly lower in calves fed a single dose (100 g of IgG) of a CR product when compared with calves fed two doses (200 g of IgG) of the same CR or 3.8 l of maternal colostrum containing 55–502.2 g of IgG in another study [95]. Additionally, these calves that received the two doses of CR had evidence of API. Similar to previous reports, the timely administration of an adequate total mass of IgG was essential to achieve API.

In one study, 100% of calves fed 150 g of IgG of a CR product developed FTPI compared with only 11% of calves fed 450 g of IgG of the same CR product [96]. In another study, serum IgG at 24 hours of age was evaluated in four groups of calves. The calves were fed either 3.79 l of maternal colostrum containing 106 g/l IgG, 3.79 l of low-quality maternal colostrum supplemented with 40 g of IgG (41 g/l IgG), a single dose of a CR containing 110 g of IgG, or a single dose of a CR containing 150 g of IgG. Calves fed maternal colostrum or low quality maternal colostrum supplemented with 40 g of IgG had a higher mean serum IgG concentration compared with calves fed the CRs (27.04, 22.33 g/l vs 12.79, 16.90 g/l, respectively) [70]. Additionally, 20% of calves fed a single dose of CR containing 110 g of IgG developed FTPI. In a report, feeding dairy heifer calves 300 g (three packages of 100 g each) of a colostrum derived commercially available licensed CR within the first 6 hours of life resulted in a mean 24-hour serum IgG of 26.9 g/l and a mean serum total protein (STP) of 6.2 g/dl [97]. CRs are effective as a substitute for maternal colostrum when administered in the first six hours of life and when provided in adequate total mass of IgG (>250 g).

Studies evaluating the use of CRs in beef calves and their efficacy providing API are lacking; however, observations

during practice in our teaching hospital indicate that the administration of at least 250–300 g of IgG from CRs during the first six hours of life are necessary to achieve API. One survey evaluated calf management practices on beef cow-calf operations and found that if a producer intervened because of inadequate suckling, 13% of those producers' first choice was to use a CR [40].

Colostrum Quality

The quality of colostrum is the most important factor in any colostrum management program. Colostrum quality refers to the IgG concentration (grams per liter) present in maternal colostrum. The IgG concentration considered optimal for maternal colostrum is >50 g/l [95]. Colostral quality can be assessed very easily on dairy farms at the time of first milking. Beef cow colostrum is easily stripped from the udder following a dystocia, as well as in times when the cow is caught in a chute to allow the calf to suckle. Assessment of colostrum quality will allow the veterinarian or producer to properly utilize or discard the colostrum based on quality test results. Cost, time, and lack of training all contribute to decreased evaluation of colostrum at the farm level. Quality of colostrum can be tested by measuring IgG concentration with radial immunodiffusion (RID), immunoassays, enzymatic assays, hydrometers (colostrometers), or the Brix refractometers [61, 65, 98–101]. Direct measurement of IgG (RID, other assays) is the most accurate method to evaluate colostrum quality and is considered the gold standard; however, this test is not feasible to do at the farm. An on-farm test needs to be quick, user-friendly, accurate, and reliable [61]. A recent report indicated that 45.1% of all dairy operations used visual appearance to estimate colostrum quality rather than a true test [39]. The colostrometer was used on 11.4% of all dairy operations in the USA and 4% of farms in Quebec, Canada [39, 102]. The Brix refractometer was used on only 4.1% of farms in the USA [39].

Using color and consistency of colostrum as indicators of quality is an inadequate and wrong assumption of the level of IgG present. The goal of assessing colostrum quality is to distinguish between poor (<30 g/l of IgG) and good (minimum >50 g/l of IgG) quality colostrum and then apply that information to develop strategies to improve the colostrum management program. The individual IgG concentration of maternal colostrum is highly variable. Studies have demonstrated that the proportion of low quality colostrum (<50 g/l of IgG) found on dairy operations after assessment of quality with RID, colostrometers, or Brix refractometers varies between 7 and 32% [61, 99, 100, 103, 104].

The colostrometer has been previously validated for the evaluation of colostrum quality [105]. Colostrometers measure the specific gravity of the colostrum and give an estimate of the amount of Ig that is present [61, 105]. Disadvantages of the colostrometer include the following: its fragility (glass); colostral temperature must be at 22 °C (72 °F); other non-Ig solids content usually affects the results; and cleaning after each use [61, 87, 105]. Use of the colostrometer is simple and personnel can easily be trained to utilize this testing modality. Common limiting factors of colostrometer use are an inadequate volume of colostrum and temperature of colostrum.

Brix refractometry is another option for on-farm evaluation of colostral quality. Refractometers measure the total solids within a solution or the refractometric index of a liquid on a Brix scale [99, 103]. Optical Brix refractometers require an individual to utilize the refractometer with the eye piece and determine at what percentage the blue lines fall. Digital refractometers give a Brix score by shining a light through the liquid and measure the index of refraction of that sample [99]. The result is displayed as a percentage. The refractometer also has disadvantages including calibration and sensitivity to light; however, Brix refractometers are less temperature sensitive than the colostrometer. They are easy to use and provide a quick estimate of IgG concentration. An optical and digital Brix refractometer were compared to the standard RID in determining IgG concentrations from maternal colostrum [99]. The mean Brix score of fresh and frozen colostrum samples using an optical and digital Brix refractometer was approximately 26% each and the correlation between the Brix refractometers and RID was moderate ($r = 0.71$ and 0.73, respectively) [99].

Results from one study demonstrated that when using a specific gravity of <1055 with the colostrometer to classify poor quality colostrum (<50 g/l of IgG), it had a sensitivity of 32% and a specificity of 97%, indicating a high chance of misclassifying a colostrum sample as adequate quality when in reality the sample was of poor quality [100, 104]. A study evaluating dairy cow colostrum quality with RID, Brix refractometer, and colostrometer found that 29.1, 14.1, and 34.3%, respectively, were below the cut point recommended for each testing modality [61]. The correlation between the RID and Brix refractometer and colostrometer was moderate ($r = 0.64$ and 0.77, respectively). Accuracy of the Brix refractometer and colostrometer was highest when the Brix reading was 23% and the colostrometer was at 80 g/l [61].

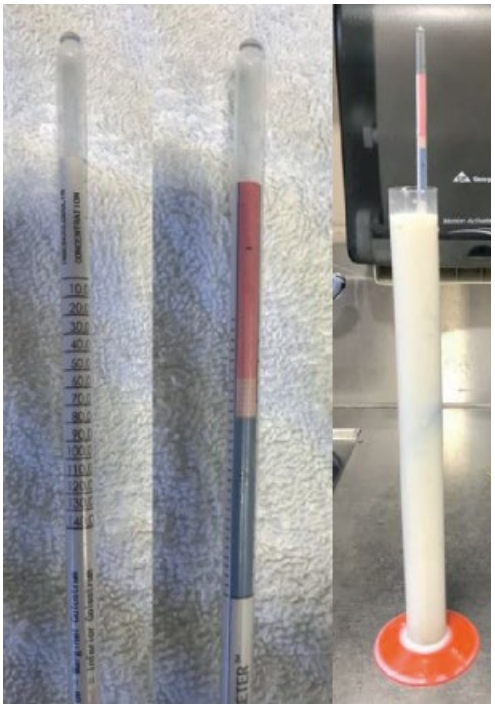
Colostral quality has been investigated using beef cow colostrum [65]. This study evaluated RID and Brix refractometry on 396 colostral samples from 92 suckler herds that included breeds such as Charolais, Belgian Blue, Blonde d'Aquitaine, and Limousin. Results

demonstrated that the mean colostrum IgG concentration was 95.9 ± 36.2 g/l. The mean Brix percentage was 26.3 ± 5.2 and the correlation between the RID and Brix refractometer was good (r = 0.8). Additionally, the authors evaluated different colostral IgG concentrations and determined the Brix percentage associated with each IgG level. For 50, 75, and 100 g/l of IgG in beef colostrum, the associated Brix (percentage) values are 22.5, 25.5, and 26.9%, respectively.

Other assays have been studied to assess colostral quality; however, their use has not been greatly implemented in the dairy industry [101, 106]. The Brix refractometer and colostrometer are the indicated methods to evaluate colostrum quality and to make decisions on storage and overall colostrum management (Figures 75.2–75.4).

Failure in the Transfer of Passive Immunity (FTPI)

Absorption of IgG and other active immune factors from colostrum are critical for the health and survival of newborn calves. Therefore assessing the levels of serum IgG during the first 48 hours of life provides an accurate prediction of future calf health and performance before and after weaning [1, 3, 5, 108]. As mentioned in previous sections of this chapter, high serum circulating levels of IgG (>10 g/l) that result from the timely consumption of an adequate volume of high quality colostrum is what defines API in dairy



1. Collect a first milking colostrum sample from a prepared udder.
2. Please sample in a graduated cylinder leaving approximately 3 cm at the top. Colostrum should be approximately 22°C or 72°F.
3. Put the colostrometer into the graduated cylinder and allow it to "float" for approximately 5 seconds.
4. Read the colostrometer. Refer to Figure 4 for recommendations.

*Images courtesy of Dr. Herris Maxwell.

Figure 75.2 Instructions on using a colostrometer. (Note instructions included in figure.) *Source:* Images courtesy of Dr. Herris Maxwell.



1. Digital reader should be clean of debris.
2. Place a drop of tap water, sterile water, or sodium chloride on the sample area.
3. Press start, the light will flash in the sample area. If the % read does not show "000", press the "zero" button.
4. Discard the water sample and lightly dry sample area with a towel.
5. Add colostrum sample to the sample area and press "Start."
6. Results will be displayed within 2 seconds. Refer to Figure 4 for recommendations.

*Images and instructions courtesy of Simon Taylor and Dr. Herris Maxwell

Figure 75.3 Instructions on using the Brix refractometer. (Note instructions included in figure.) *Source:* Images courtesy of Simon Taylor and Dr. Herris Maxwell.

Colostrum Quality	Colostrometer/Hydrometer	Brix Refractometer (%)	Recommendation for Use
Good	Green (>60–90 g/L)	>23%	Use of Store *
Fair	Yellow (30–50 g/L)	18–22%	Use as a second feed^
Poor	Red (<30 g/L)	<17%	Discard

* Store under refrigeration for up to 48 hours and if not used by this time then should be frozen for up to 1 year.
^ This quality colostrum should only be used if the calf has received good colostrum and the appropriate amount at the first feed.

Figure 75.4 Assessment of colostral quality, measurements, and recommendation for use. *Sources:* [61, 107].

calves. During the last 30 years or so, serum IgG levels <10 g/l have been used as the cut-off to define FTPI in the dairy industry 30, 66, 109, 110, the, last, within, years.[39]. Dairy calves with serum IgG concentrations of less than 10 g/l when sampled within one to seven days of age are classified as having FTPI. This classification standard was based on significant reduction of preweaning mortality observed in young dairy calves with serum IgG levels >10 g/l between 24 and 48 hours of age [7, 12, 56, 109, 110]. The adoption and implementation of this standard by producers and veterinarians during recent decades resulted in a significant improvement in dairy colostrum management programs and reduction of calf mortality. Today, more than 90% of heifer calves on US dairy operations meet or surpass the current serum IgG standard (10 g/l) [39, 66] and preweaning dairy heifer mortality was reduced from 10.8% in 1992 to 6.4% in 2014 [39, 111]. During the same period and despite overall improvement in the transfer of passive immunity, the rate of preweaning dairy calf morbidity did not significantly change (36.1% in 1991 vs 33.9% in 2014) [39, 110, 111]. Studies suggest that higher serum IgG levels than traditionally recommended are associated with reduced dairy calf morbidity. Results from one study in which 175 neonatal dairy heifers were stratified by serum IgG levels at 30–60 hours of life demonstrated that calves with serum IgG levels ≥15 g/l had lower preweaning rates of respiratory disease and diarrhea compared with calves with lower IgG levels [1]. The authors of another study where 1290 dairy heifers were stratified in categories by IgG and STP at 48 hours of life reported significant reduction of preweaning morbidity and mortality associated with the serum levels of IgG and STP. The reduction of health events occurred in the heifers with serum IgG levels of 20–25 g/l and STP of 5.8–6.3 g/dl compared with other groups [108].

In contrast to the <10 g/l of serum IgG used in dairy calves to define FTPI, the beef industry has adopted a cut-off of >24 g/l of serum IgG to define API, a value much higher than the dairy industry [3, 5]. In one study, the association between serum IgG levels and preweaning morbidity/performance was evaluated in 1568 crossbred beef calves. Results from this study demonstrated significant reduction of morbidity and mortality when serum IgG in calves was ≥24 g/l at 24–72 hours of age. Calves with serum IgG <24 g/l were 1.6 times more likely to become ill and 2.7 more likely to die before weaning. Additionally, increases in individual BW (>3.35 kg) were reported at 205 days of age in calves with serum IgG levels >27 g/l [5]. Results from another study where serum IgG levels at 24 hours were contrasted with preweaning morbidity and mortality in 935 beef calves from 153 different cow-calf farms demonstrated that the odds of treatment and death were greater for calves with serum IgG <24 g/l. [3] Similarly, results from a study involving 1392 beef calves determined that to significantly reduce morbidity and mortality, the optimal 24-hour serum IgG and STP cut-off values needed to be >24 g/l and >6.0 g/dl, respectively [112]. Based on literature reports in dairy calves and data from beef calves, there is strong evidence that the dairy calf standard established to define optimal transfer of passive immunity (>10 g/l) does not reflect the beneficial effects of higher serum IgG levels on reduction of calf morbidity and enhanced health and performance.

A study that involved several US dairy calf experts from government institutions, academia, industry, and private sectors proposed new standards for optimal values of serum IgG, STP, and percentage Brix associated with API in dairy calves based on data collected during the 2014 Dairy NAHMS report [6]. Results from this study demonstrated significant reduction of preweaning dairy calf morbidity at different levels of serum IgG between one and five days of life. Four classification categories, instead of the previous two (adequate vs failure), of serum IgG and corresponding STP and percentage Brix values that reflect different risks of preweaning morbidity and mortality were recommended for implementation in US dairy operations (Table 75.1).

Table 75.1 Herd-level categories and optimal values of serum IgG, STP, and Brix, with recommended proportion of calves for each category.

	Categories of transfer of passive immunity			
Value/proportion at 24–48 hours	**Excellent**	**Adequate**	**Reasonable**	**Failure**
IgL (g/l)	>25	18–24.9	10–17.9	<10
STP (g/dl)	>6.2	5.8–6.1	5.1–5.7	<5.1
Brix (%)	>9.4	8.9–9.3	8.1–8.8	<8.1
Calves (%)	>40	~30	~30	<10

Source: Adapted from [6].

Factors Associated with Transfer of Passive Immunity in Calves

Several factors from the dam, calf, general management, and environment can affect adequate consumption of colostrum and absorption of IgG and other immune factors increasing the risk of FTPI.

Colostrum Quality

Genetic factors such as breed and management factors such as the prepartum diet, pooling practices, and time of collection have a direct influence on the concentration of IgG in colostrum [45, 113, 114]. Holstein cows tend to produce higher volumes of colostrum compared with any other breed. A previous study suggested that Holsteins produced colostrum with mean IgG concentrations significantly lower than other dairy breeds due to a dilution effect [113]. However, studies demonstrated that the average IgG concentration in colostrum from Holstein cows in the USA is >68 g/l, which is higher than the standard for good quality dairy colostrum [66, 103].

Colostrum IgG concentration in beef breeds is usually >95 g/l and values of >100 g/l are considered of adequate quality [65, 115]. One study reported that FTPI was four times more likely in calves born to cows that had a higher frequency of expression of a defective mammary gland Fc-IgG receptor [116]. The expression of the defective Fc receptor was found to be greater in Gelbvieh, Charolais, and Simmental cows in another study [117]. Although in the past it was believed that colostrum from first-calf heifers had lower IgG concentrations compared with colostrum from older cows, more recent studies have demonstrated that colostrum quality of beef and dairy first-calf heifers is adequate and should not be discarded without testing [66, 118].

The effect of the prepartum diet on colostrum IgG concentration in dairy cows is controversial and it has been difficult to establish. Some studies have suggested that restricted protein and energy diets during the last 100 days of gestation in beef cows resulted in lower IgG levels in colostrum and greater risk of FTPI in their calves [114, 119, 120].

Pooling of raw maternal colostrum from different cows is a common practice on US dairy operations. Results from one study indicated that only 25% of pooled maternal colostrum meets the quality standards for IgG concentrations (50 g/l) [79, 103]. Pooled colostrum usually overrepresents poor quality maternal colostrum, increasing the risk of FTPI [79].

Individual variation in colostrum IgG concentrations in addition to the above-mentioned factors introduce a persistent variability in the total mass of IgG fed to each calf, particularly on farms that feed a fixed volume (i.e. 4 l) of maternal colostrum. Ingestion of an adequate total mass of IgG is the single most important factor in acquiring API. The greater the total mass of IgG consumed by the newborn calf, the higher the level of IgG in serum at 24 hours [121]. Variation in IgG concentrations of maternal colostrum could not only affect the presentation of FTPI but also reduce the transfer of specific immunity [122].

Colostrum Cleanliness

Providing the newborn calf with colostrum free from bacteria reduces the presentation of FTPI [42, 123]. Results from some studies suggest that contamination of colostrum with bacteria disrupts absorption of IgG through the intestinal epithelium, reduces the efficiency of absorption of IgG, and increases the risk of neonatal septicemia [13, 42]. Direct competition through non-selective pinocytosis, binding of free IgG in the intestinal lumen, and breakdown of IgG by bacterial enzymes have been proposed as potential mechanisms by which bacterial contamination of colostrum affects IgG absorption and increases the risk of FTPI [42, 123–125]. Results from some studies have demonstrated that heat treatment of maternal colostrum significantly reduces bacterial contamination, reduces transmission of

colostral-borne pathogens, and increases IgG absorption in calves [66, 84, 125, 126].

Time of Colostrum Feeding

Delayed colostrum feeding or delayed suckling increases the risk of FTPI. Results from one study demonstrated that feeding the same source of maternal colostrum to dairy calves at 4, 6, and 12 hours of age compared with feeding within 2 hours of age reduced serum IgG at 24 hours between 10 and 15, 25–30, and 40–50%, respectively [72]. Similarly, a study in beef cow-calf operations suggested that calves that failed to suckle maternal colostrum within the first 4 hours of age had increased odds of FTPI and veterinary treatment compared with calves that successfully suckled within the first 4 hours of age [52]. Results from another study demonstrated that IgG at 24 hours in serum was reduced by 0.32 g/l for every hour delay in colostrum feeding after birth [66]. A prior report indicated that for every 30-minute delay in colostrum feeding after birth, a reduction of 2 g/l of IgG was observed in serum at 24 hours of age [127].

Physiological Status of the Calf

Calves that experience calving stress (dystocia) and different degrees of hypoxia, hypercapnia, and metabolic acidosis have reduced serum IgG levels at 24 hours and increased risk of FTPI compared with healthy calves born from normal parturition [3, 128, 129]. Additionally, factors such as hypothermia, weak suckle reflex, failure to withdraw the tongue after birth, and failure to stand during the first hour of age have been associated with FTPI in dairy and beef calves [52, 58, 130–134].

Method of Feeding

On commercial dairy operations, letting the calf naturally suckle its dam usually results in a higher incidence of FTPI due to inability of suckling enough volume and adequate mass of IgG [135]. The use of a nipple bottle to deliver a high volume of good quality colostrum is effective and preferred by many dairy producers [110, 136]. However, in some instances the use of an esophageal feeder is required to guarantee the delivery of an adequate total mass of IgG and prevention of FTPI. When administering larger volumes (>3 l) of maternal colostrum through an esophageal feeder, calves achieve API and AEA is usually unaffected [137]. Some certified organic dairy operations and all beef cow-calf operations rely on the mothering ability of the dam and the ability of the newborn calf to stand and suckle to acquire colostrum in a timely manner. One study reported an FTPI (<10 g/l) prevalence of 30.8% in 156 calves from 20 organic dairies that allowed calves to suckle their dams to acquire colostrum [138]. In contrast, the prevalence of FTPI (<24 g/l) in 47 beef calves that naturally suckled first-calf heifers (dams) after birth in a single cow-calf operation was 13.6% and the mean serum IgG at 24 hours was >30 g/l [118]. This suggests that when concentration of IgG is adequate in maternal colostrum, suckling the dam results in serum levels of IgG higher than expected. Poor udder conformation can negatively affect adequate suckling of colostrum. Results from one study demonstrated that poor udder conformation (pendulous, teats close to the ground, teats too large or too small) prolongs teat-seeking behavior and delays time of colostrum ingestion in calves [139].

Diagnosis and Monitoring of Transfer of Passive Immunity

Survival and overall health of newborn calves during their first months of life are determined in great proportion by their ability to acquire API. For this reason, monitoring the level of serum IgG as a predictor of future calf health through on-farm diagnostic methods such as STP and serum Brix is an important management strategy to prevent morbidity and mortality associated with FTPI. Assessment of STP and serum Brix in calves is a strategic herd management tool that allows producers to adjust their colostrum management program. Monitoring STP and serum Brix is a common practice on dairy operations because management offers an inherent advantage of sampling calves at 24–48 hours. Beef cow-calf operations are in need of on-farm strategies to monitor transfer of passive immunity in their calves. In beef calves, producers commonly rely on subjective methods such as visualization of suckling and palpation of the calf's abdomen at 24 hours to determine adequate suckling and transfer of passive immunity [52, 58].

The single radial immunodiffusion (SRID) test is considered the gold standard laboratory test to evaluate serum concentrations of IgG in calves and determine success or FTPI [140]. Additional laboratory tests to determine serum IgG in calves include turbidimetric immunoassays (TIA) and enzyme-linked immunosorbent assay (ELISA) tests. The correlation of TIA and ELISA with SRID is good ($r = >0.9$) and these laboratory tests can be used as an alternative to the SRID test [141]. Laboratory testing can be expensive and unpractical for producers because results usually are not readily available to make decisions on management or treatment of individual cases of FTPI. For this reason, on-farm methods to assess transfer of passive immunity in calves that deliver immediate results are preferred by

producers and veterinarians. The most common on-farm methods to evaluate transfer of passive immunity in calves include refractometry (STP and digital serum Brix), whole blood glutaraldehyde coagulation, and sodium sulfite/zinc sulfate turbidity tests. Here we will focus on STP and serum Brix, as these are the on-farm diagnostic methods most commonly used by producers in the USA.

Serum Total Protein (STP)

Measuring serum concentration of proteins is a reliable method to evaluate transfer of passive immunity because IgG makes most of these proteins. Results from previous studies have demonstrated a good correlation between STP and serum IgG evaluated by SRID [142–144]. Additionally, associations between higher STP at 24 hours and decreased calf morbidity and mortality rates have been reported [4, 110, 145, 146]. When using a cut-off value of serum IgG of 10 g/l, STP values of 5.2–5.5 g/dl collected between 1 and 7 days of age demonstrated a good to very good correlation with SRID (r = 0.74–0.97) [143, 144, 147]. With the new standard recommendations on transfer of passive immunity for dairy calves and available data from beef calves, the goal of STP to reach API is >5.8 g/dl for dairy and >6 g/dl for beef calves, respectively [3, 6]. It is important to mention that for individual calves, STP could underestimate true serum IgG levels and careful interpretation of results is necessary in these cases before intervention [73, 148]. Results from some studies suggested that for calf-level decision-making, STP testing is optimum between 24 and 72 hours of age, whilst for herd-level decision-making, STP is good up to 9 days of age [147–151]. As a herd-monitoring tool, less than 10% of the calves should have an STP <5.1 g/dl between 24 hours and 5 days of age, as described in Table 75.1 [6]. Accurate interpretation of STP results is important to adjust colostrum management. Factors such as red cell contamination, hemolysis, dehydration, and inflammation could falsely elevate serum protein concentrations in calves and produce inaccurate readings [144, 148]. Proper maintenance of the refractometer is also essential to obtain accurate results and make sound management decisions. The application surface of the refractometer should be cleaned between each use with 70% alcohol, followed with recalibration of the instrument with distilled water (every 10–20 samples). A reading of 1.000 indicates adequate calibration.

Serum Brix

The level of total solids in milk, milk replacers, colostrum, and serum can be assessed using digital Brix refractometers. Several studies have evaluated the correlation of serum Brix at 24 hours of age and serum IgG by SRID to determine the presence of FTPI in newborn calves [147, 152, 153]. Results from one study of 1756 serum samples collected from calves between one and five days of age demonstrated that a serum Brix value of 8.4% predicted API in 98.3% of the calves when using a serum IgG of 10 g/l [153]. Other studies have equally demonstrated a good serum Brix and SRID IgG correlation (r = 0.74–0.93) when using a serum Brix value of ≥8.4% and serum IgG of 10 g/l [152, 154, 155]. The serum Brix value expected to predict API in dairy calves based on the new standards is ≥8.9% [6]. Similar to STP, the serum Brix is a convenient method to assess FTPI at the herd level, but it could underestimate true serum IgG levels in about 45% of individual samples [153]. As a herd-monitoring tool, less than 10% of the calves should have a serum Brix of <8.1% between 1 day and 5 days of age, as described in Table 75.1 [6]. Factors that affect STP reading as well as maintenance and calibration are similar for proper and accurate function of digital Brix refractometers [156].

Management Strategies to Prevent FTPI

Reduction of dairy- and beef-calf morbidity and mortality is achieved through absorption of high levels of IgG from high quality maternal colostrum very soon after birth. Colostrum management programs should ensure production of high quality colostrum, appropriate colostrum collection, and storage of high quality and pathogen-free colostrum. Delivery of a volume of colostrum that contains an adequate mass of IgG in a timely manner to newborn calves should also be achieved.

Production of High Quality Colostrum

Nutrition and vaccination programs during the prepartum period directly affect the production of high quality maternal colostrum. Results from studies on the effects of prepartum nutrition (last 100–60 days of gestation) in beef and dairy cows on the concentration of IgG in maternal colostrum and serum IgG levels in their calves are controversial [119, 120, 157]. Two studies in beef cows suggested that although protein and energy restriction during the last 90–100 days of gestation in beef cows did not have an effect on colostrum IgG, serum IgG levels at 24 hours were lower in calves that suckled colostrum from cows on the restricted diets [119, 120]. A proceedings report indicated that low energy diets in beef cows 30 days before calving resulted in lower colostrum IgG concentrations and a greater risk of FTPI in their calves [114]. In contrast, colostrum and calf

serum IgG concentrations were not different in a study where high or low energy diets were administered to dairy cows during the dry period and early prepartum [157]. Results from another study suggested that meeting energy requirements of dairy cows during the dry period resulted in greater colostrum IgG compared with colostrum IgG from cows on a diet that exceeded dry period energy requirements [158]. Management recommendations for dietary composition of prepartum dairy and beef cows should follow the National Research Council standards.

High serum IgG levels at 24 hours in calves do not always translate into higher levels of specific antibodies against common enteric and respiratory pathogens that cause disease on each individual farm. Results from one study suggested that FTPI was responsible for an increased presentation of preweaning respiratory disease in dairy calves in 21% of the cases only. Instead, calves with higher colostrum derived bovine respiratory syncytial virus (BRSV) and bovine herpesvirus 1 (BHV-1) antibody titers had lower odds of developing preweaning respiratory disease compared with calves with lower BRSV and BHV-1 titers [159]. Results from studies in dairy and beef cattle demonstrated that vaccination with two doses of a multivalent killed virus (KV) respiratory vaccine during gestation results in higher specific antibodies against BVDV 1, BVDV 2, BHV-1, and BRSV in maternal colostrum. Additionally, calves that suckled colostrum from vaccinated cows had higher initial BVDV 1, BVDV 2, BHV-1, and BRSV antibody titers compared with cows that suckled colostrum from unvaccinated cows [118, 160, 161]. Similarly, several studies documented that vaccination during late gestation with multivalent KV enteric vaccines increases specific immunity against calf diarrhea and respiratory disease-causing pathogens in maternal colostrum and newborn calves [162–165]. Vaccination programs to increase specific immunity in maternal colostrum in cows and transfer of specific antibodies to calves should respond to individual farm needs and individual farm calf-pathogen ecosystems.

Clean Collection of Colostrum

Contamination of maternal colostrum during collection can become a potential route of early transmission of infectious pathogens to newborn calves. Essential steps to avoid colostrum contamination during collection are thorough udder preparation and sanitization, milking with clean and sanitized equipment into a clean and sanitized bucket, and transferring colostrum into a clean and sanitized storage or feeding device. Feeding calves with colostrum contaminated with bacteria results in decreased IgG absorption and increased risk of FTPI [42, 123–125]. Colostrum for calves should contain less than 100 000 colony-forming units (cfu)/ml total plate count and less than 10 000 cfu/ml total coliform count [125]. Immediately after collection, colostrum should be tested, fed to calves, or properly stored because if left at room temperature significant bacterial grow and proliferation occurs.

Storage of High Quality and Pathogen-Free Colostrum

Newborn calves should only receive colostrum with adequate concentrations of IgG and free from infectious pathogens such as coliforms, *Mycobacterium avium subsp paratuberculosis*, *Salmonella* spp., *M. bovis*, BLV, and BVDV. In ideal circumstances, colostrum for calves should come only from cows that have tested negative to the previously mentioned pathogens. Colostrum with IgG concentrations >50 g/l is considered of adequate quality; however, IgG levels of individual colostrum samples should be tested with a hydrometer (colostrometer) or a digital Brix refractometer, as discussed in the section "Colostrum Quality." Maternal colostrum with >23% Brix or >60–90 g/l of IgG on the colostrometer (Figure 75.4) can be fed immediately to newborn calves. When fresh colostrum will not be administered immediately after collection, it should be refrigerated at 4 °C for 12–24 hours or frozen at −20 °C for up to 1 year, ensuring repeated freeze–thawing does not occur [126, 166]. Heat treatment of maternal colostrum at 60 °C (140 °F) for 60 minutes is an alternative to eliminate important infectious pathogens such as *E. coli*, *Salmonella enteritidis*, and *M. bovis* and to significantly reduce the risk of exposure to *M. avium* subsp. *paratuberculosis* while conserving IgG levels [166–168]. Additionally, heat treatment of colostrum reduces overall bacterial contamination and increases aborption of IgG in the calf [66, 125]. Heat treatment of colostrum should occur after IgG testing and before freezing and storage. When ready to use, stored (frozen) colostrum should be thawed avoiding water temperatures >60 °C (140 °F) to prevent inadvertent denaturation of IgG [126, 166].

Delivery of an Adequate IgG Mass in a Timely Manner

To achieve new standards for transfer of passive immunity in dairy calves or API in beef calves, administration of colostrum should consist of a single feeding of at least 300 g of total IgG mass during the first 2–4 hours of age. Alternatively, administration of two feedings of 200 g of total IgG mass each with the first feeding during the first 2–4 hours of age and the second feeding within the first 6 hours of age is recommended [6]. If testing of maternal colostrum for IgG concentrations is not possible, administration of a volume

equal to 10–12% of BW (3–4 l) during the first 2–4 hours of age with an additional feeding of at least 2 l within the first 12 hours of age is recommended for dairy calves. Results from one study demonstrated that feeding dairy calves with 4 l of maternal colostrum at 0 hours and an additional 2 l at 12 hours resulted in higher serum IgG levels (31.1 g/l) at 24 hours compared with calves fed only 2 l at 0 hours and another 2 l at 12 hours (23.5 g/l) [169]. In beef calves, monitoring calving ease and ensuring the newborn has a strong suckle reflex during the first 15 minutes of birth and is standing and suckling within the first hour of age are essential to prevent FTPI [52, 58]. In beef calves born from a dystocia or prolonged calving, with a weak suckle reflex, swollen tongue or poor tongue withdrawal, and inability to stand during the first hour of age, assisting colostrum intake is necessary to prevent FTPI. One option is placing the dam in a chute and allowing the calf to suckle for 15–20 minutes. Alternatively, the cow can be milked thoroughly and maternal colostrum administered to the calf through a nipple bottle or an esophageal feeder. At the teaching hospital, we usually administer 2 l of maternal colostrum or a CR product during the first 2 hours of age and if natural suckling has not occurred within the next 4–6 hours, the calf receives an additional 2–4 l dose of colostrum.

Summary

The administration of an adequate mass of IgG from maternal colostrum or a CR product in a timely manner to newborn dairy and beef calves is a key management strategy to guarantee reduced morbidity and mortality and increase productive performance. Although several factors can affect individual colostrum quality, it is essential to establish an on-farm testing protocol for maternal colostrum to identify colostrum with adequate and poor IgG levels and make appropriate colostrum use and storage decisions. On beef cow-calf operations where a colostrum management program is usually uncommon, monitoring of calving and early evaluation of the calf's vigor after delivery are important factors to make decisions on artificial colostrum feeding. Assessing the level of transfer of passive immunity in newborn calves through on-farm calf diagnostics such as STP and serum Brix refractometry is recommended to make management adjustments to consistently achieving API in the calf herd. Highly competitive performance and productive results of dairy or cow-calf beef operations start with the delivery of clean, pathogen-free, and IgG-loaded maternal colostrum to newborn calves.

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Section IV

Assisted and Advanced Reproductive Technologies

Technological Advances that Enhance the Quantity and Quality of Spermatogenic Production

76

Utilization of Genomic Testing for the Selection of Desirable Traits in Cattle

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Introduction

Genomic technologies have been widely adopted within the beef and dairy industries and their popularity and utility continue to increase. Applications of genomic technologies in the beef and dairy industries include tests for specific causal mutations (largely genetic abnormalities but also Mendelian traits like coat color), parentage testing, and genomic testing for production traits. Inclusion of genomic testing results is now common within genetic evaluation, and this is generally the recommended application of these data for quantitative production traits. This chapter will explain how to utilize basic genomic testing results for Mendelian traits and how genomic analyses are useful in the context of bovine fertility-related traits, and provide a look ahead to the emerging genomic technologies that may impact beef and dairy production, particularly as it pertains to animal reproductive efficiency.

Background

To fully understand the contributions of genomic selection and genomic technologies to the bovine industry, it is very important to have a general understanding of basic genetic principles. Thus, we will review some of those principles here.

The physical characteristics displayed by an animal are known as a phenotype. Broadly, phenotypes are the result of genotype, gene interactions, and (often) environmental factors. A genotype is the combination of alleles found in an organism, and will consist of two alleles (i.e. AA, Aa, or aa) for each location in the genome (locus). Alleles are essentially differences in the nucleotide sequence between organisms, which often lead to differences in phenotypes. Single base changes in the DNA (sort of like typos in the DNA sequence) are known as single nucleotide polymorphisms [SNPs], which are currently the most commonly utilized genetic marker in livestock. As technology has improved, so has our ability to examine large numbers of SNPs at one time. The ability to analyze up to several hundred thousand SNPs at once on products known as SNP chips has facilitated the development of livestock selection at the genome level [1].

Genomic technologies have revolutionized the way in which veterinarians, researchers, and producers understand and evaluate cattle health and productivity. Genomics now contributes to a variety of products and information sources that can be utilized by producers in making selection decisions. Veterinarians can also use genomics for diagnostics. This chapter will review many of the common uses of genomic technology in the beef and dairy industries.

Simple Mendelian Traits

Gregor Mendel laid the foundation for modern genetics back in the 1860s [2]. His recognition of the principles of inheritance allowed for the discovery of what geneticists have termed simply inherited traits, or Mendelian traits. Simply inherited traits are traits in which the phenotype is often determined by a single mutation and is not influenced by the environment. Thus the phenotype will be a direct result of which two alleles were inherited at that locus. Frequently, one allele will be dominant and conceal the other recessive allele in cases where an animal is heterozygous (has two different types of alleles at a locus) [3]. Although the exact mode of inheritance of these traits varies (many are autosomal recessive, but some, like hyperkalemic periodic paralysis in horses, are autosomal dominant), simply inherited traits can

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

easily be selected for in livestock species if a genomic test exists. Examples of simply inherited traits in the beef and dairy industry include the Extension locus that is responsible for black or red coat pigmentation, the POLLED locus that controls the presence or absence of horns, and loci associated with genetic abnormalities [4] (Table 76.1). Figure 76.1 outlines examples of selecting for simple Mendelian traits that are inherited as completely dominant.

One of the most important uses of genomic technologies is for the tracking and management of recessive genetic abnormalities. Recessive alleles only result in an affected individual when two copies of the allele are present (i.e. they are homozygous). However, heterozygote carriers also possess a single recessive allele, which they can pass to their progeny even though the heterozygote does not exhibit the phenotype associated with the recessive allele. This phenomenon is why genomic testing to identify and manage carrier animals is so useful, because they cannot be identified by their phenotype.

Many alleles corresponding to genetic abnormalities are lethal, but there are plenty of exceptions, as compiled in Table 76.1. Most lethal alleles are recessive, as shown in the example in Figure 76.2, and thus two copies of the allele result in death. Dominant lethal alleles are exceptionally rare, because only one copy of the allele is necessary to cause death. Therefore dominant lethal alleles only persist for conditions that are late-onset, after the individual has already reproduced. There are additional classes of lethal alleles as well. Conditional lethals are alleles that cause fatality in response to an environmental stimulus [6]. The most well-known example of a conditional lethal disorder is favism in humans. Affected individuals are deficient in glucose-6-phosphate-dehydrogenase, an enzyme responsible for proper red blood cell functionality, but only experience severe hemolytic anemia when in contact with specific stressors like fava beans or aspirin [7]. Furthermore, alleles can be semilethal in which most affected offspring die but a few individuals in the generation survive [8]. While numerous examples of recessive lethal alleles exist (see Table 76.1), there are no known examples of conditional lethal or semilethal alleles in the beef or dairy industry.

Fortunately, most known genetic abnormalities in cattle are recessive. Thus, as long as ranchers do not breed two heterozygous animals together, they should never observe an affected calf. Figure 76.3 demonstrates this point. Although it may seem logical to eradicate the condition entirely by culling any animals that are carriers of a disease allele, there are several cogent reasons to manage the recessive allele instead. The most important of these reasons are to maintain genetic diversity and to continue to make forward strides in genetic improvement. With genomic testing, producers do not need to indiscriminately cull genetically superior animals that carry a deleterious allele. Instead, producers can utilize genomic testing to slowly reduce the frequency of the allele in the population without sacrificing genetic gain or producing affected calves.

Parentage Testing

Genomic testing can also be used to establish parentage. Parentage verification can be useful for tracking bloodlines in herd books, breed recognition, or proof of pedigree for registration. Parentage testing can be used by producers to confirm an animal's sire or dam [9], but sire verification is most common. Parentage testing involves genotyping offspring and any potential parents to find inconsistencies in the inheritance of genetic markers between the progeny and possible sires or dams. An example of parentage testing can be seen in Figure 76.4. It is based on one of the foundational principles of genetics: that a diploid animal receives one copy of a randomly segregating allele from each parent.

Parentage testing excludes animals that cannot be parents of the offspring in question, rather than definitively determining parentage. Parentage panels will typically have 96 SNP markers, but using as many as 400 SNP markers has been proposed. When performing parentage testing, genotyping errors are accounted for by allowing one exclusion (inconsistent inheritance) out of all the markers in the panel without disqualifying the animal as a potential parent. Two to three exclusions would result in a retest of the sample to rule out contamination, unsatisfactory DNA quality, or poor genotyping results. More than three exclusions would result in elimination of that animal as a potential parent of the progeny in question. As such, it is important that progeny testing abides by the following general principles:

1) Be consistent in the selection of marker panels. All animals should be genotyped with the same panel. For example, if an older animal is genotyped on a microsatellite panel, it will need to be re-genotyped on an SNP panel if the progeny are genotyped on an SNP panel.
2) It is essential to include all possible parents when parentage testing. Do not include animals that could not possibly be the sire or dam. Including too many potential parents increases the risk of receiving ambiguous results or misidentifying parentage of the individual(s) of interest, but failing to include a potential parent can result in an incorrect result. In some cases, potential sires can be eliminated without genotyping if inheritance of simple Mendelian characteristics is impossible (such as a red bull mated to a red cow producing a black calf).

Table 76.1 Allelic control of common bovine Mendelian genetic traits.

Phenotype	Abbr.	Affected breed(s)	Mode of inheritance	Lethal/ non-lethal	Monitored by breed?
Arachnomelia	—	Braunvieh, Simmental	AR	Non-lethal	No
Arthyogryposis multiplex (curly calf syndrome)	AM	Angus	AR	Lethal	Yes
Bovine blood coagulation factor XIII deficiency	F13	Wagyu	AR	Non-lethal	Yes
Bulldog dwarfism (chondrodysplasia)	BD	Dexter	AR	Lethal	Yes
Chediak-Higashi syndrome	CHS	Wagyu	AR	Non-lethal	Yes
Chondrodysplasia	CHO	Angus	AR	Lethal	Yes
Claudin 16 deficiency	CL16	Wagyu	AR	Non-lethal	Yes
Contractual arachnodactyly (fawn calf)	CA	Angus	AR	Non-lethal	Yes
Coat color	CC			Non-lethal	Yes
Red/black		Many beef breeds Limousin, Gelbvieh, Dexter, Hereford, Charolais	Black AD Red AR		
Dilution	DL		Incomplete dominance		
Dun	DN		AR		
Developmental duplication	DD	Angus	AR	Non-lethal	Yes
Deficiency of urine monophosphate synthase	DUMPS	—	AR	—	—
Digital subluxation	DS	—	AR	—	—
Erythrocyte membrane protein band III deficiency (spherocytosis)	Band 3	Wagyu	AR	Often lethal	Yes
Hypotrichosis	HY	Hereford	AR	Nonlethal	Yes
Factor XI deficiency	F11	Wagyu	AR	Non-lethal	Yes
Freemartinism	FM	All	—	Sterile female	Yes
Idiopathic epilepsy	IE	Hereford	AR	Non-lethal	Yes
Alpha-mannosidosis	MA or MAN	Red Angus	AR	Lethal	Yes
Beta-mannosidosis		Salers	AR	Lethal	Yes
Myostatin		Belgian Blue, Piedmontese, Limousin	—	Non-lethal	No
Neuropathic hydrocephalus	NH	Angus	AR	Lethal	Yes
Osteopetrosis	OS	Angus, Herford, Red Angus, Simmental	AR	Lethal	Yes (Red Angus)
Pulmonary hypoplasia with anasarca	PHA	Shorthorn, Dexter, Maine-Anjou	AR	Lethal	Yes
Horned/polled	Polled			Non-lethal	Yes
Holstein-Friesian/Jersey	Pf	Holstein, Jersey, Angus, Brahman, Blonde d'Aquitaine, Charolais, Dexter, Hereford, Limousin, Santa Gertrudis, Shorthorn, Simmental, Tropical Composite	AD		
Celtic	Pc		AD		
Tibial hemimelia	TH	Galloway, Shorthorn, Maine-Anjou	AR	Lethal	Yes

[AR] Autosomal recessive mode of inheritance.
[AD] Autosomal dominant mode of inheritance.

Example 1a: The Extension Locus

	B	***b***
B	*B*/*B* 	*B*/*b* 
b	*B*/*b* 	*b*/*b* 

Notation

B/*B* = animals with this genotype express the black coat color phenotype and only possess black coat color alleles (B)

B/*b* = animals with this genotype express the black coat color phenotype, but are carriers of the red coat color allele (b)

b/*b* = animals with this genotype express the red coat color phenotype

Coat color is a commonly used simple Mendelian test used in the beef industry. Here we have a heterozygous black bull mated to a heterozygous black female. The resulting offspring are 25% homozygous black, 50% heterozygous black, and 25% homozygous red. The hidden, recessive red allele in heterozygous black parents explains how two black animals can produce a red calf.

Example 1b: The POLLED Locus

	P	***P***
p	*P*/*p* 	*P*/*p* 
p	*P*/*p* 	*P*/*p* 

Notation

P/*P* = animals with this genotype express the polled (hornless) phenotype and possess only polled alleles (P)

P/*p* = animals with this genotype also express the polled phenotype, but are carriers of the horned allele (p)

p/*p* = animals with this genotype express the horned phenotype

The POLLED locus is another example of a simple Mendelian trait important in the beef and dairy industry. Polled animals are often desired because the natural absence of horns prevents the need for dehorning, which is a painful procedure. Horns are often dangerous for the humans managing the cattle as well as other cattle in the herd. Genetically selecting for the polled phenotype allows producers to improve both handler safety and animal well-being. It is important to note that as long as we mate any given animal to a homozygous polled animal, we should never observe horned offspring.

Figure 76.1 Examples of phenotypic outcomes for completely dominant Mendelian traits.

3) Dam genotypes are helpful, but not essential, in sire verification. Often, it may not be economical to genotype the dam. As shown in the example above, parentage can be determined without complete information on the dam. However, if both parents must be verified (such as in the case of pedigree verification or embryo parent verification), the dam must be genotyped.
4) It is more difficult to determine parentage when the potential sires are related. Related sires and dams share the same alleles, so it will be more difficult to definitively determine parentage.

Following these guidelines will help ensure that parentage testing is utilized correctly and with the greatest economy.

Genetic Evaluation of Production Traits

Genotype data can also be utilized in genetic evaluation for production traits. Continuous traits, which are also called quantitative traits, include most economically relevant

	N	***PHA^D***
N	***N/N*** 	***N/PHA^D*** 
PHA^D	***N/PHA^D*** 	***PHA^D/PHA^D*** 

Notation

N/*N* = animals with this genotype express the normal phenotype

N/*PHA*^{D} = animals with this genotype express the normal phenotype, but are carriers of PHA

PHA^D/*PHA*^{D} = animals with this genotype express the PHA phenotype and are aborted or stillborn

Pulmonary hypoplasia with anasarca (PHA) is an autosomal recessive genetic defect that can affect multiple breeds of cattle, including Maine-Anjou, Shorthorn, and Dexter. It leads to fetal death in calves that are homozygous recessive for the mutation [5]. Animals with the defect have inadequate lung development and severe swelling throughout the body from excessive fluid. Carriers of PHA appear phenotypically normal. When crossing two PHA carriers, we would expect that 25% of the progeny would express the PHA phenotype and perish before, or shortly after, parturition.

PHA is particularly dangerous because affected calves often swell so severely that they also contribute to an increased risk of dystocia for the dam [5]. Occasionally, PHA-affected calves are aborted early in gestation, which is similar to the effect of embryonic lethal alleles (discussed later in this chapter) and can make it difficult to identify the presence of the PHA allele in a population in the absence of genomic testing.

Figure 76.2 An example of recessive lethal alleles.

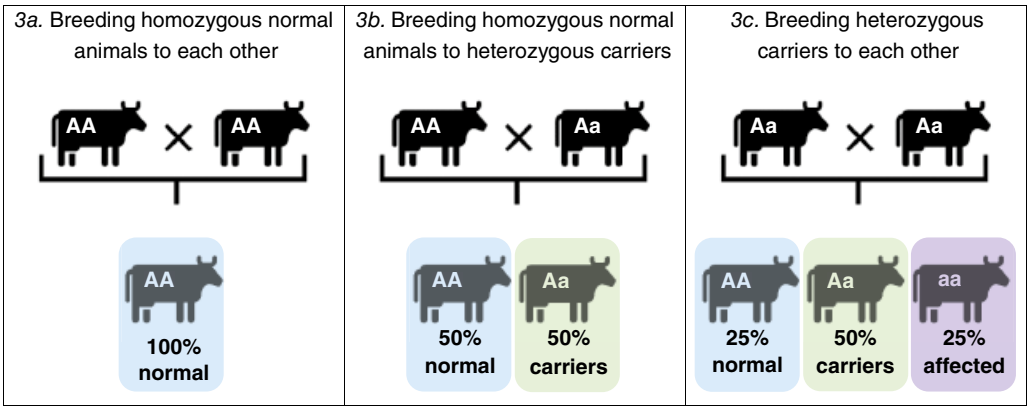
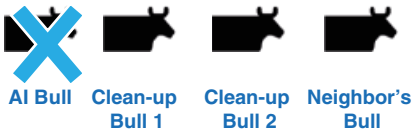


Figure 76.3 Managing breeding decisions can diminish the effect of lethal and deleterious alleles.

	Locus					
Animal	***A***	***B***	***C***	***D***	E	***F***
Calf	*A/A*	*b/b*	*C/c*	*D/d*	*E/E*	*F/F*
Dam	-	-	-	-	-	-
AI Bull	*a/a*	*b/b*	*C/c*	*D/d*	*E/e*	*f/f*
Clean-up Bull 1	*A/A*	*B/B*	*C/C*	*D/d*	*E/E*	*f/f*
Clean-up Bull 2	*A/a*	*B/b*	*C/C*	*d/d*	*E/e*	*F/f*
Neighbor's Bull	*A/A*	*B/b*	*c/c*	*D/d*	*e/e*	*F/F*

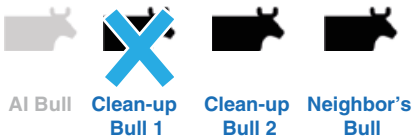
LOCUS A

At the A locus, the calf will receive an *A* allele from its dam and another *A* allele from its sire. Even though we do not know the mother's genotype at this locus, we can exclude the AI bull who does not have an *A* allele.



LOCUS B

At the B locus, the calf will inherit a *b* allele from its dam and another *b* allele from its sire. Since clean-up bull 1 is homozygous *BB* and the calf is homozygous *bb*, clean-up bull 1 can be ruled out as a possible sire.



LOCUS C AND LOCUS D

At these loci, the calf is heterozygous. Since we do not know the dam's genotype, none of the sires can be eliminated from the list of potential sires.



LOCUS E

The calf must have inherited an *E* allele from both the dam and the sire, which means that because the neighbor's bull does not have an *e* allele to give, he can be excluded.



LOCUS F

At this locus, clean-up bull 2 and the neighbor's bull are the only potential donors of the calf's second *F* allele, but from the alleles at locus E, we have already excluded the neighbor's bull, leaving clean-up bull 2 as the only remaining possibility.



From these data, you would conclude that **clean-up bull 2 is the most likely sire of this calf**.

Figure 76.4 Example for the use of parentage testing: You are a cattle producer and recently purchased a promising heifer. You artificially inseminate the heifer and turn her out on native rangeland with two clean-up bulls in a multisire pasture. Unfortunately, your neighbor has poor-quality fences, so his bull jumps the fence and spends some time with your cattle. You are now trying to register the calf, but you need to determine which bull is the sire, so you genotype both the calf and the sires on a standard parentage panel with 96 SNP markers. The results for six loci are shown. Based on the information given in the chart, which bull is the most likely sire?

production traits like weaning weight, milk production, marbling, and age at first calving. Quantitative traits are polygenic, meaning that they are influenced by multiple alleles or mutations throughout the genome. Continuous traits show a range of phenotypic variation due to both genetic and non-genetic factors. The difference between continuous traits and discontinuous traits can be seen in Figure 76.5. Genetic factors encompass those that are both stably inherited (additive effects of alleles) and those that are not (gene combination values that arise due to cross-breeding). Non-genetic factors include environmental effects such as nutrition, health status, and weather.

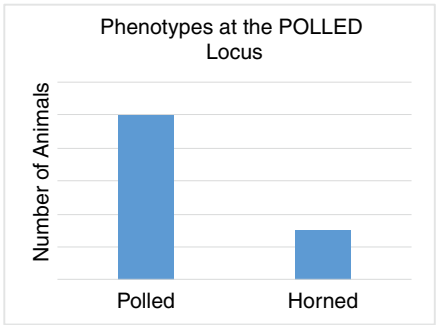
Genetic evaluation is designed to quantify the additive genetic merit of individuals and reflects only the portion of genetic variance that is stably inherited [10]. In other words, it estimates the additive effects of alleles as a numeric value that describes the merit of an individual as a potential parent for the next generation. Genetic evaluations are frequently used by breeders as selection tools to achieve a variety of breeding objectives and are performed using best linear unbiased prediction (BLUP). The ultimate goal is to provide cattle producers with the tools necessary to increase the production of food and fiber products from their livestock animals. BLUP incorporates data from all of the sources outlined in Figure 76.6 to produce genomically enhanced expected progeny differences (EPDs) or predicted transmitting abilities (PTAs) [11]. These mixed models account for both fixed effects (such as sex, age, year) and random effects (such as animal). Ultimately, BLUP has become the foundational model for the genetic evaluation of domestic animals [12].

Cattle breeders gauge genetic merit by using EPDs in the beef industry or PTAs in the dairy industry. EPDs and PTAs represent the best estimates of genetic merit based on all available phenotypic, pedigree, and genomic information for each animal. EPDs and PTAs represent the predicted differences between the future average progeny performance of individuals for a specific trait. These values are generally not comparable across breed associations, although there are exceptions to this rule. Historically, EPDs and PTAs were generated without genomic information. However, due to the industry-wide utilization of genotyping in the beef and dairy industries, these data are now included in the genetic evaluation for most major beef and dairy breeds.

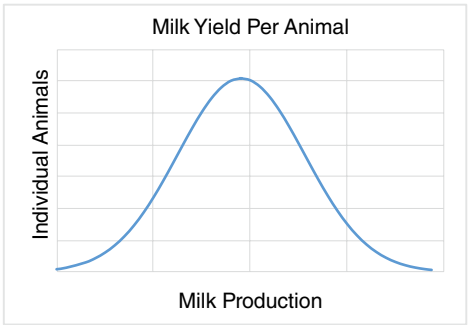
The genomic data included in genetic evaluation are derived from high-density genotyping, in which a large number of SNPs can be genotyped at one time (often called SNP chips) [13]. This type of genotyping is so useful because, depending on the panel, it can include information on lethal alleles or other markers of interest (like coat color), in addition to information that can be used to augment genetic evaluation for production traits. High-density SNP panels are more affordable than ever, which enables cattle producers, veterinarians, and researchers to more efficiently and cost-effectively use these technologies.

Advantages of Genomic Selection

Estimating breeding values with phenotypic records and pedigree information provides producers with reliable estimates of genetic merit [14], but is most accurate for older, widely used animals that have large numbers of progeny in the breed association database. This is especially challenging for traits recorded late in life, such as longevity, where animals are at an advanced age before highly accurate genetic predictions can be generated. Incorporating genomic data into genetic prediction helps build accuracy on these animals at a younger age.



Discontinuous traits are expressed as a limited number of possible phenotypes. For example, the POLLED locus in cattle is a discontinuous trait because the polled phenotype does not overlap with the horned phenotype.



Milk yield is a continuous trait because individual cows will produce differing amounts of milk. As demonstrated above, continuous traits, like milk yield, typically follow a normal distribution.

Figure 76.5 The difference between continuous and discontinuous traits.

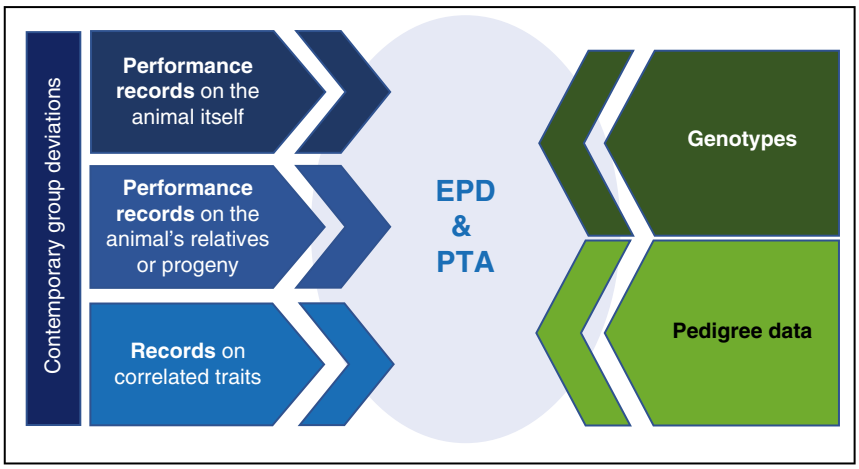


Figure 76.6 The data that go into the calculation of EPDs and PTAs.

Initially, marker assisted selection was the only available method for utilizing genomic information on animals for selection. This created multiple sources of (often contradictory) information that could not be combined into a single metric for selection, which was confusing for producers. Marker assisted selection has been surpassed by the use of genomic selection [11]. Development of SNP-chip genotyping platforms has made genomic selection more cost-effective and tractable for breeders, at least within the seedstock sectors of the industry [11].

In December 2007, the Illumina BovineSNP50 BeadChip (50K SNP chip) was commercially released with a set of 54 001 SNPs. SNP chips have become the most widely used assays in both the beef and dairy industries [14]. Lower density (LD) SNP assays have been developed, as well as a higher density chip, the Illumina 778K SNP BovineHD [15] assay. Unfortunately, even though higher density allows for greater precision for fine-mapping quantitative trait loci (QTL), it does not substantially increase prediction accuracy [16]. Therefore mid-density SNP chips have become the gold standard in the industry. Often, animals are genotyped at a lower density (often called LD genotyping), and higher density genotypes are imputed using software to "fill in" the missing genotypes [17]. This process works well and is widely used, though some ambiguity exists in regions of the genome where recombination occurs.

Genetic evaluations are performed by beef and dairy breed associations as often as weekly in order to incorporate new data collected by breeders (such as newly collected phenotypes or genomic testing results). The addition of genomic data into prediction of genetic merit has led to an increased level of genetic gain. In the beef industry, inclusion of genomic data has resulted in increased growth and carcass quality [18] (such as improved ribeye area and marbling scores). In the dairy industry, genomics has transformed selection of younger animals, thereby decreasing the generation interval and increasing genetic gain [19]. Genomic selection increases genetic gain in highly heritable traits, but the inclusion of genomic information has also been shown to benefit lowly heritable ones, like those associated with reproduction and fertility [20]. In US dairy cattle, the implementation of genomic selection has led to a 50–100% increase in annual genetic gain for highly heritable traits and a 300–400% increase in genetic gain for lowly heritable traits [21]. Genomic selection is now used extensively across the USA and has made the livestock selection process much more efficient and effective.

Achieving Genetic Gain Through Genomics

When employing artificial selection for production traits, there are multiple factors that can dictate the amount of genetic gain that can be achieved. Genetic gain is represented with the following equation:

$$\Delta G = \frac{i \times r \times \sigma_A}{L}$$

In this equation, ΔG represents the rate of genetic gain within a population, i is the selection intensity, r is the accuracy of the breeding value of the animal, σ_A is the additive genetic standard deviation of the specific trait, and L is the generation interval [22]. Rate of genetic gain can also be expressed as the rate of genetic change per unit of time and can be denoted as Δ_{BV}/t, where Δ_{BV} is the change in breeding value of an animal and t is the unit of time used to measure the generation interval [23]. The inclusion of genomic data into genetic prediction can impact genetic gain in several ways: by improving selection intensity due to easier identification of the most superior animals, increase in accuracy of the breeding value (EPD/PTA), and decreases in the generation

interval due to the ability to identify superior animals earlier in life and cycle generations more quickly. A decrease in generation interval is especially meaningful not only because it is in the denominator of the equation, but also because the generation interval in cattle is longer compared to other species. Genomically enhanced EPDs and PTAs, coupled with current reproductive technologies like sexed semen, multiple ovulation and embryo transfer (MOET), *in vitro* embryo production (IVF), embryo genotyping, and cloning, have the ability to dramatically affect genetic gain [22].

Genomic Evaluations

In the past, genomic evaluations were performed using a complex, multistep method that required a combination of the traditional BLUP evaluation and a summation of estimated marker effects for each trait. Both pieces of information were subsequently blended together using a variety of methodologies that differed among different breed associations to develop the final genomically enhanced genetic merit prediction. Because of the fact that these two pieces of information could sometimes be contradictory (which was confusing for producers) and the methodology was cumbersome, effort was invested in developing "single-step" methodologies for incorporation of genotypes into genetic evaluation.

The development of single-step methods for inclusion of genomic data into genetic evaluation has been a major improvement. Current implementations provide a benefit to genetic prediction for both genotyped and non-genotyped animals. Single step increases accuracy of predictions [24], especially for young genotyped animals [25]. Due to its efficiency and simplicity, single step has become the favored methodology for genomic evaluations in beef and dairy breeds [26]. Current implementations of single-step methodologies can be categorized into either single-step genomic BLUP (single-step GBLUP) or marker effects models that use the Biometry Open Language Tools (BOLT) software. We will briefly overview these two methodologies.

GBLUP

Genomic BLUP incorporates marker information by augmenting the relationship matrix for genotyped animals, instead of simply using pedigree [27]. This additional information assists to correct pedigree errors, accounts for shared alleles from animals beyond the recorded pedigree, and accounts for the fact that animals may be more or less related than expected due to the sampling of alleles inherited from their parents. An example of this can be seen in Figure 76.7 [28]. Though we make assumptions about the number of shared alleles in the genome based on pedigree, the actual relationship between two animals can fall among a distribution. For example, full siblings will, on average, share 50% of their genome, so the relationship between them using pedigree is 0.5. However, SNP data might indicate that they share a greater or smaller portion of their genome, which is reflected in the relationships in a GBLUP. The distribution of grandparent relationship is shown in Figure 76.8 [28]. We expect the grandparent relationships to be 0.25, but with SNP data we can more accurately account for the sampling of alleles, and thus the values will vary (with the average being approximately 0.25). In 2014, the University of Georgia released a program called BLUPF90 that will perform single-step GBLUP [29]. This program is widely used by many different breed associations for genetic evaluation, including the Holstein Association USA and the American Angus Association [26]. While BLUPF90 does have the capability to weight markers more heavily (such as those that are known to have large effects on a trait), current implementations are unweighted.

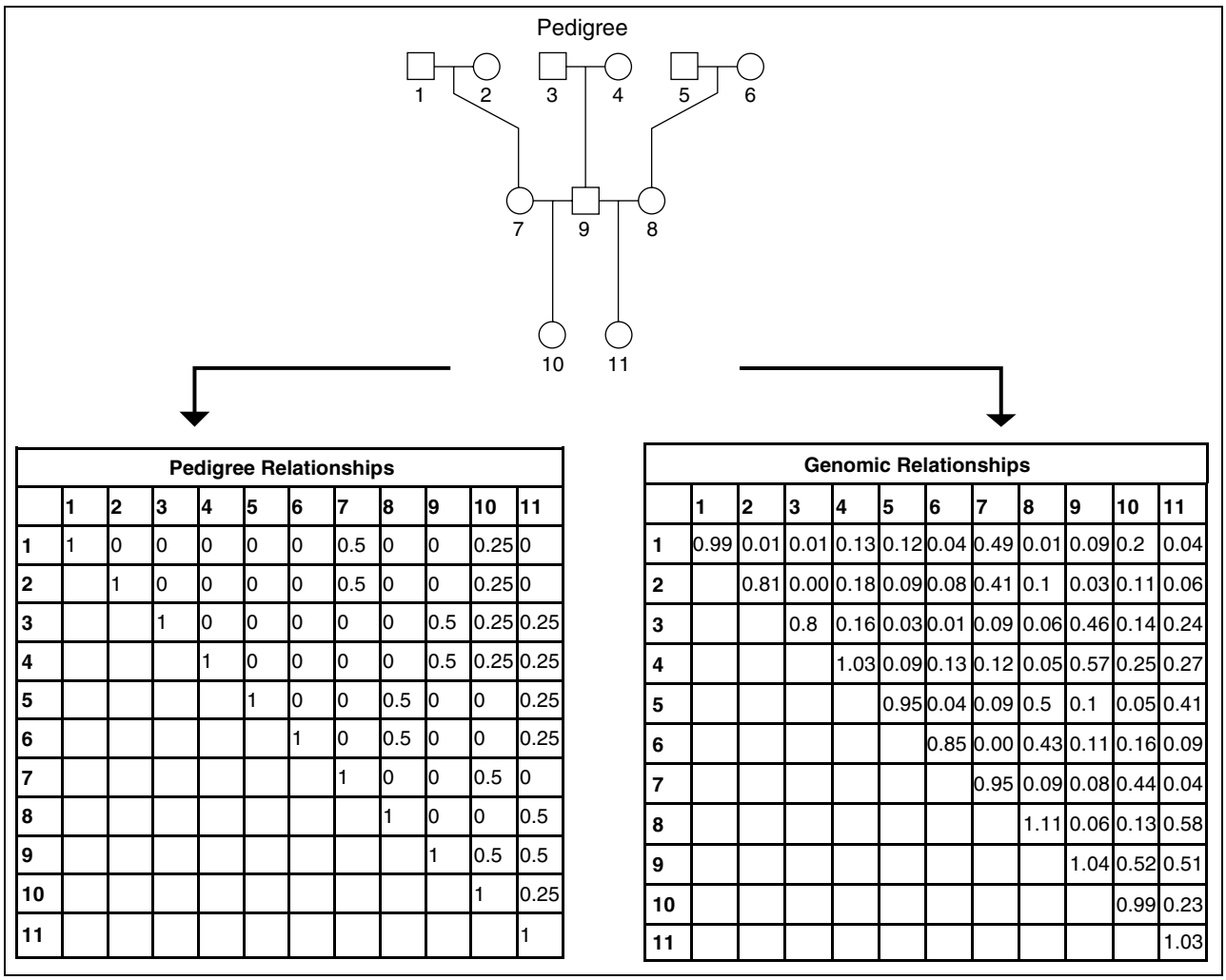
BOLT

Another option for single-step genomic prediction is BOLT, which was developed by Theta Solutions LLC and introduced as a result of a collaboration between the American Simmental Association (ASA) and Dr. Bruce Golden and Dr. Dorian Garrick. BOLT software offers the ability to fit a variety of models and increases accuracy of genetic predictions [30]. In contrast to single-step GBLUP, BOLT allows certain DNA markers, usually highly associated with the traits in the model, to play a larger role in the prediction of the genetic merit of an animal. BOLT also calculates true accuracy, rather than estimating prediction accuracy. However, since the "approximation" method used to find accuracy usually results in overestimation of the value, the true accuracy calculated by BOLT for animals can be lower than their previous accuracy [31]. BOLT is currently used by International Genetic Solutions (IGS) [31] and the American Hereford Association (AHA) [32].

Use of Genomics in the Cattle Industry

Dairy

Before genomic selection was introduced, dairy producers and AI companies used progeny testing to gather enough data on potential AI sires to provide producers with a higher accuracy PTA. Progeny testing is a system where elite females are chosen and bred to specific sires to produce bull calves that will then be test mated to a population [33].



Pedigree Relationships

	1	2	3	4	5	6	7	8	9	10	11
1	1	0	0	0	0	0	0.5	0	0	0.25	0
2		1	0	0	0	0	0.5	0	0	0.25	0
3			1	0	0	0	0	0	0.5	0.25	0.25
4				1	0	0	0	0	0.5	0.25	0.25
5					1	0	0	0.5	0	0	0.25
6						1	0	0.5	0	0	0.25
7							1	0	0	0.5	0
8								1	0	0	0.5
9									1	0.5	0.5
10										1	0.25
11											1

Genomic Relationships

	1	2	3	4	5	6	7	8	9	10	11
1	0.99	0.01	0.01	0.13	0.12	0.04	0.49	0.01	0.09	0.2	0.04
2		0.81	0.00	0.18	0.09	0.08	0.41	0.1	0.03	0.11	0.06
3			0.8	0.16	0.03	0.01	0.09	0.06	0.46	0.14	0.24
4				1.03	0.09	0.13	0.12	0.05	0.57	0.25	0.27
5					0.95	0.04	0.09	0.5	0.1	0.05	0.41
6						0.85	0.00	0.43	0.11	0.16	0.09
7							0.95	0.09	0.08	0.44	0.04
8								1.11	0.06	0.13	0.58
9									1.04	0.52	0.51
10										0.99	0.23
11											1.03

Figure 76.7 Pedigree and genomic relatedness. *Source:* Adapted from [10].

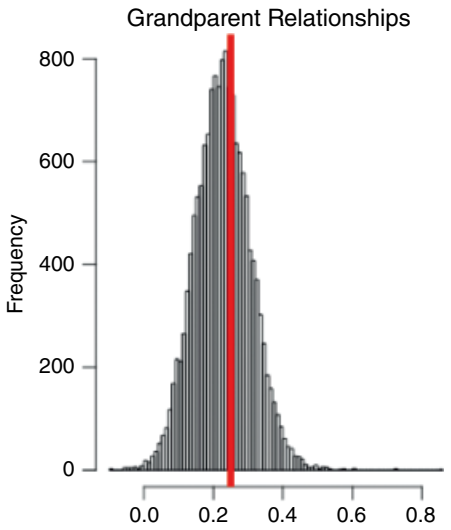


Figure 76.8 Grandparent relationship. *Source:* Based on [10].

The daughters of these bulls were then bred and first lactation performance recorded. With progeny testing, the bull's PTA for milk production can achieve an accuracy of 75–80% in approximately 64 months [33]. Since most economically important traits in dairy operations are sex limited and the generation interval is long, it takes numerous years to obtain highly accurate genetic predictions with this method. Progeny testing systems have decreased in popularity due to the ability to produce genomically enhanced PTAs on young prospective herd sires. Producers and artificial insemination (AI) companies are now capable of identifying genetically desirable animals using PTAs enhanced by genomic data at a younger age than by progeny testing [34].

Genomic evaluations were first developed for the Holstein and Jersey breeds in 2009 [14]. Other breeds soon followed, with genomic evaluations released for Brown Swiss in late 2009, Ayrshire in 2013, and Guernsey in 2016 [14]. Today, the release schedule for genomic evaluations depends on

breed association policies. For example, the Holstein Association USA provides customers with preliminary (weekly) genomic evaluations, interim monthly genomic evaluations, and official genetic evaluations. Preliminary evaluations are different than the monthly evaluations because they are made available shortly after a producer sends in a sample, only include genomic-PTAs [35], and are provided to producers and breeders who plan to make early culling decisions. However, the use of these specific evaluations is prohibited for marketing the animals [14]. The monthly genomic evaluations are when haplotypes or genetic test results are provided to the producer [36]. Official genetic evaluations are released three times a year (April, August, and December) [35]. For the Jersey breed, genetic evaluations are updated the first Tuesday of each month [37]. In addition to the USA, other countries like New Zealand, Australia, Canada, and numerous Europeans countries incorporate genomic information into their genetic evaluations [11].

Utilizing genomic information in selection provides the opportunity for a surge in genetic gain within the dairy industry. Greater availability of genotypes on both bulls and females paired with abundant phenotypes on these animals has led to a substantial increase in the accuracy of genomic prediction for production and fertility traits in the dairy industry [11]. The increase in accuracy of estimating the genetic merit for individuals at a young age provides producers numerous advantages when it comes to breeding, purchasing, marketing, and producing high-quality, elite livestock for the industry [14].

Beef

All major US beef breed associations conduct genetic evaluations to estimate EPDs. In the past, genetic evaluation was constrained within breed. However, 12 breeds including the associations for Chianina, Gelbvieh, Limousin, Maine-Anjou, Red Angus, Simmental, Shorthorn, Canadian Simmental, Canadian Gelbvieh, Canadian Limousin, Canadian Angus, and Canadian Shorthorn have begun collaborating to develop a multibreed database through IGS [38]. The IGS breeds as well as many breeds with individual evaluations (including American Angus, Hereford, Brangus, and Beefmaster) offer their members and producers genomic information to aid in selection decisions in the form of genomic enhanced EPDs (GE-EPD).

For the dairy industry, the availability of low-density chips caused whole herd genotyping to increase in popularity, and a large majority of those genotypes are obtained from females instead of males due to the structure of the industry [14]. In the beef industry, the number of genotyped females is dramatically less than males. To increase numbers of genotyped females, some breeds have developed programs that offer their customers an incentive for testing entire herds or progeny groups. For example, the ASA developed a program called Cow Herd Roundup (CHR). The program encourages whole herd DNA testing by offering lower prices on tests and additional savings if producers also provide cow weights with either body condition scores or hip heights [39].

Genotyping and genomic selection have become popular among seedstock producers as both a selection and marketing tool. These practices are far less accepted among commercial producers, mostly due to cost and return on investment. It has been suggested that DNA testing could improve accuracy of replacement female selection in a commercial operation [40]. However, cow replacement rates are lower; thus, genetic gain is achieved at a slower rate than other sectors. These factors contribute to the more difficult economies of using genomic technologies in the commercial sector. In a simulation study that estimated economic returns and costs for four different genetic evaluation scenarios (two included purebred cattle and the other two included purebred and commercial cattle), the 30-year cumulative net present value was determined per commercial calf. Scenarios were separated by type of information (phenotype, genotype, and pedigree) included in the genetic evaluation. In each scenario that incorporated commercial cattle information, genotypes improved accuracy of selection candidates but increased the time to breakeven for the operation by six to seven years [41]. Even if return on investment for genotyping their own progeny is more challenging for commercial producers, they can still utilize genomic technologies by purchasing herd bulls with GE-EPDs, which are more accurate than traditional EPDs for young sires [42].

Male Fertility

Improving fertility in livestock is extremely important to increase the production of food and textile products and the economic efficiency of livestock production. Male fertility is defined as the capability of the male to copulate and produce offspring. Male fertility traits in genetic evaluations have generally focused on scrotal circumference in the beef industry and sire conception rate (SCR) in the dairy industry. However, greater emphasis on male fertility traits in the future is certainly possible and could include numerous semen quality and quantity traits. Currently, AI centers and veterinarians performing bull breeding soundness examinations (BBSEs) record many semen quantity traits, including ejaculate volume, concentration of spermatozoa in ejaculate, and number of spermatozoa in ejaculate. Other semen quality traits include spermatozoa motility, percentage of normal spermatozoa, and spermatozoa abnormalities.

The phenotypes documented by AI centers and BBSE records provide the phenotypic structure for a genetic evaluation of these traits, provided that a framework for the delivery of these data to breed associations were to be developed. Utilizing genomics to find regions of the genome that impact male fertility traits has slowly gained popularity. While the beef industry has reported few SNPs associated with genes affecting fertility traits, the dairy industry has discovered regions of the genome that could be utilized to identify mutations or genes associated with male fertility traits.

Scrotal Circumference

Scrotal circumference is included in some beef breed association genetic evaluations including Angus, Hereford, Limousin, and Charolais. It was initially used in genetic evaluations due to its ease of collection in yearling bulls and relationship with semen quantity and growth traits [43]. However, inconsistent correlations between scrotal circumference and traits like daughter pregnancy rate (DPR) [44], calving interval [45, 46], and average daily gain have been reported [47]. Although it is arguable whether scrotal circumference is an indication of female fertility due to some conflicting results in the literature [48, 49], scrotal circumference is currently the only male fertility-related trait in genetic evaluations of beef breeds. It is a moderately heritable trait (0.36–0.56) [43, 50–54]. Interestingly, as age increases from 12 to 24 months, the heritability of scrotal circumference remains moderate, indicating that age does not greatly impact its heritability [54].

Few studies have reported correlation estimates between scrotal circumference and semen quality traits. It has been reported that as scrotal circumference increases, so does spermatozoa motility [52–54]. As scrotal circumference increases, the percentage of normal spermatozoa has also been shown to increase [51, 54] and the percentage of abnormalities present to decrease [52, 53]. Additional correlations have been reported, but these estimates are contradictory and have high standard errors associated with them.

Sire Conception Rate

In 2008, the United States Department of Agriculture (USDA) proposed a national AI dairy bull fertility evaluation. The SCR evaluation replaced the estimated relative conception rate (ERCR) which was used from 1986 to 2005 to measure dairy bull fertility. The model used to evaluate SCR includes the level of inbreeding of the bull, the level of inbreeding of the embryo from the mating, the age of the bull, the AI center the bull was collected at, the year of collection, and the effect of the bull itself [55]. However, it is difficult to evaluate SCR using AI conception data because bulls with inferior semen quality are already eliminated from the population. Semen collected from AI sires must meet strict quality measurements to be frozen. If a sire's collection has low concentration or motility, the collection is discarded, thereby eliminating the potential variation that could have been captured with low-quality semen collections [56, 57]. The dairy industry has led the cattle industries in identifying genomic regions associated with male fertility traits, and as a result has identified several genes potentially linked to SCR [58–61].

The following traits are not included in any genetic evaluation within the beef or dairy industries but are important candidates for the evaluation of male fertility in future EPD and PTA development efforts.

Semen Production Traits

Several studies have reported semen volume to have a moderate heritability [62–65] (0.18–0.26) in dairy AI bulls. Few studies have estimated the heritability of semen volume in beef bulls, but the currently available heritabilities are calculated with data obtained when bulls undergo BBSEs and are low with high standard errors [66, 67].

Conversely, concentration has been estimated to have a low (0.13–0.20) [50, 65, 67, 68] to moderate (0.26–0.52) heritability [63, 64, 66]. Breed differences and small sample sizes likely account for some of the variance in heritability estimates, but additional research with larger datasets is warranted.

Number of spermatozoa is calculated by multiplying volume and concentration together, which helps to explain why the heritability is similar to heritability estimates for volume and concentration. Number of spermatozoa has been estimated to have moderate heritability (0.22–0.38) [50, 63–65, 68] in both beef and dairy systems as well as when calculated with data from either AI bulls or BBSE data.

Few genes have been associated with semen production traits. Studies with Jersey and Holstein bulls identified genes associated with sperm motility [58, 69]. A study in Austria with Fleckvieh bulls identified four genes associated with number of spermatozoa [70].

Semen Quality Traits

Variable motility heritability estimates and inconsistent collection methods for motility traits make it difficult to compare previously published results. Motility heritability estimates are published under a variety of monikers, such as spermatozoa motility, progressive motility, motility score, and mass activity. Generally, heritability estimates for these motility traits are low to moderate. Although some studies suggest motility is a lowly heritable trait, the majority of heritability estimates that use AI center data report that motility is moderately heritable (0.30–0.43) [63–65, 71, 72].

Likewise, low heritability estimates have been noted for the percentage of normal spermatozoa [68], but most studies

have reported moderate heritability estimates for percentage of normal spermatozoa (0.35–0.47) [54, 67, 73]. This variability in reported estimates indicates that more research on this trait should be conducted to better understand the potential for incorporating percentage of normal spermatozoa into genetic evaluation.

Few studies report heritability estimates for spermatozoa abnormalities, but current estimates suggest that the percentage of abnormalities has a low to moderate heritability (0.19–0.35) [52, 53, 67, 72], depending on if the study evaluates abnormalities by developmental stage (primary or secondary) or specific abnormality. The different reporting systems do not seem to cause great variation in the estimate.

Moreover, few studies have reported genetic correlations among semen quality traits. Reported correlations are inconsistent and have large standard errors, which makes it difficult to determine whether selection on any of these traits would cause unintended changes in another.

While there is a lack of genomic information pertaining to semen quality traits, genes influencing the development of spermatozoa and the ability to fertilize an egg have been discovered [58, 60, 61, 69, 74]. The dairy industry has remained at the forefront of these genomic discoveries; however, the beef industry has additionally begun to search for genes associated with male fertility traits.

The lack of bull fertility studies, paired with the need to increase the efficiency of cattle production, drives the need for more male fertility research. Bull fertility in both the beef and dairy industry could be improved by selection, as these traits are generally moderately heritable. The addition of genomic data into these evaluations would likely bolster traditional selection efforts, as it would provide the same benefits seen for currently evaluated traits. AI centers provide the opportunity to collect bull fertility data on heavily used and highly influential sires, which could then be incorporated into genetic evaluation systems. Additionally, reporting data from routine BBSEs to breed associations could be a complimentary avenue to facilitate the implementation of male fertility EPDs or selection indices. If beef and dairy cattle producers had the capability to make selection decisions for improved fertility based on genetic merit, it would save time and resources as a result of the greater production efficiency and increased profitability.

Female Fertility

Genetic evaluation for fertility in females is relatively new for both the dairy and beef industries. The dairy industry began genetic evaluations for female fertility in the early 2000s, with the beef industry following [75]. Prior to selection for female fertility, the dairy industry observed a steep decline in fertility for over half a century. Selection emphasis was focused on economically important production traits, such as milk yield, which resulted in correlated responses that negatively impacted fertility (i.e. genetic gains in other traits would often lead to reduced fertility) [75, 76]. Although selecting for production traits negatively impacts fertility traits when not jointly considered, the development of properly weighted indexes can limit or constrain the impact on fertility when selecting for production yield [77].

In response to declining fertility, the dairy industry began to vigorously focus on the development of selection tools for fertility traits [75] and it has been successful in making progress in female fertility (Figures 76.9 and 76.10) [78]. Recently, the beef industry has also developed selection tools for female fertility, which has led to an increase in heifer pregnancy (HP) and sustained cow fertility

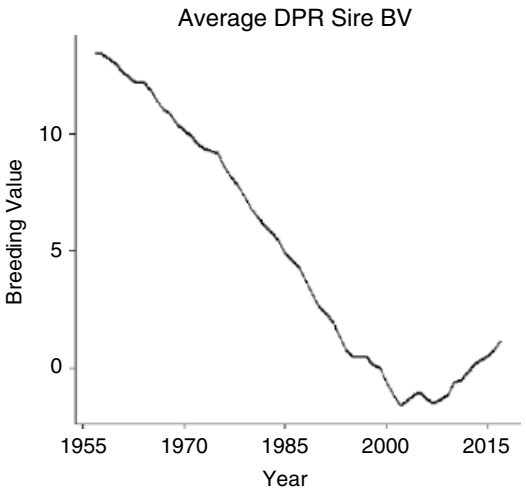


Figure 76.9 Average sire BV for daughter pregnancy rate (DPR) from 1957 to 2017 in Holsteins; selection began in the early 2000s. *Source:* Based on [78].

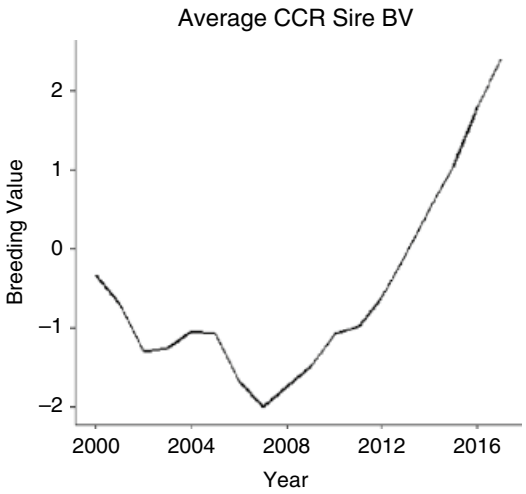


Figure 76.10 Average sire BV for cow conception rate (CCR) from 2000 to 2017 in Holsteins. *Source:* Based on [78].

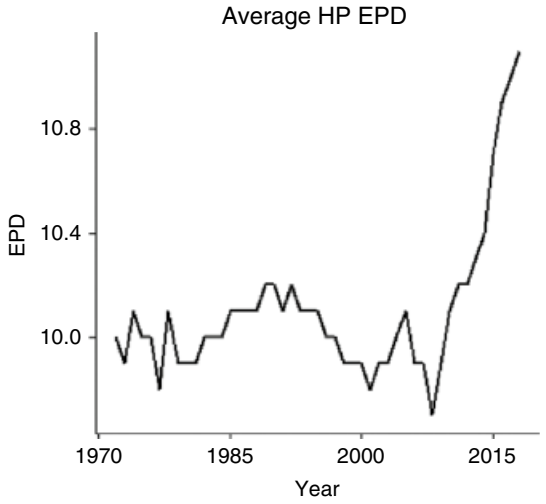


Figure 76.11 Average HP EPD in Angus from 1972 to 2018. *Source:* Based on [79].

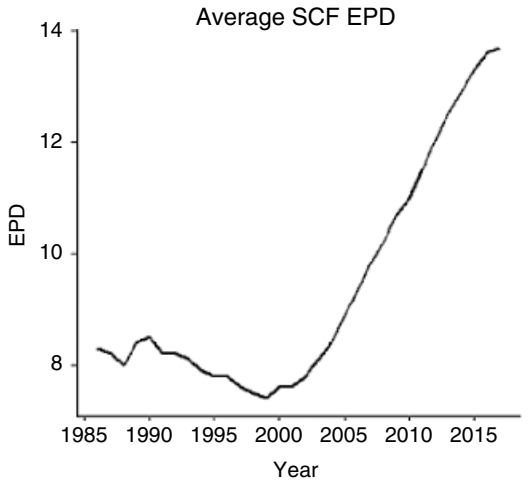


Figure 76.12 Average sustained cow fertility (SCF) EPD in Herefords from 1986 to 2017. *Source:* Based on [152].

(Figures 76.11 and 76.12), which followed declines from around 1990 to 2000. With the development of genetic evaluations for fertility traits and subsequent selection pressure in both the beef and dairy industries, the decline in average EPDs and PTAs for various fertility traits has largely reversed [75, 76, 78].

There are numerous traits that could contribute to the evaluation of female fertility. No formal definition or trait is universally favored within the industry, but traits encompassing various components of conception, pregnancy, and calving have been utilized in the research community. Phenotypes in genetic evaluation are based on producer-submitted data. Thus, the traits that could be developed for national genetic evaluation are limited to traits that can be easily and cost-effectively recorded by producers. Female fertility traits often require substantial numbers of records due to their low heritability, and complete sets of phenotypic records for an entire herd are desirable. Therefore, breed associations often provide incentives for whole herd reporting (WHR), which is especially valuable for fertility-related traits. WHR programs, which are recommended by the Beef Improvement Federation, require producers to submit data on all animals regardless of registration status. Adopting WHR programs improves genetic evaluation, leading to more accurate EPDs and PTAs [80], which are in the best interest of both the producer and the breed association. Standardizing the data collection efforts for female fertility traits may alleviate some of the concerns around availability of phenotypic information for evaluation [81]. Here we will review a variety of female traits, their use in the beef and dairy industries, and their suitability for genetic evaluation and genomic selection.

Conception Traits

Scrotal Circumference

Scrotal circumference tends to be highly heritable (Table 76.2) and has been utilized in the beef industry in Angus, Charolais, Hereford, and Limousin genetic evaluations [79, 152–154] (Table 76.3). Scrotal circumference is thought to serve as an indicator of female fertility due to an inconsistent, but potentially low to moderate genetic correlation with HP [82, 162, 163]. However, the correlation seems to come from underlying genetic effects influencing puberty rather than directly influencing fertility [81, 82]. In this case, selection would increase HP due to more heifers reaching sexual maturity at the time of breeding. It is also unclear as to whether these effects translate into cow pregnancy and fertility [77].

First-Service Conception Rate

First-service conception rate is a binary trait (success or failure) expressed as a probability of success. It emphasizes selection on females becoming pregnant to the first insemination and was designed to select for animals breeding earlier in the breeding season. Animals that conceive earlier, calve earlier, leading to increased weaning weights. They also have more time for uterine involution to take place before the next breeding season, increasing the odds the heifer will be ready to breed in the next breeding season [123]. First-service conception rate is similar to calving to first insemination, a trait used in the dairy industry. Interestingly, a wide range of heritability estimates have been generated (Table 76.2).

Table 76.2 Summary of heritability estimates (h^2) for commonly used reproduction traits in beef cattle.

Trait and h^2	References
Age at first calving	
<0.10	[49]^{T}, [82]^{T}, [83]^{C}, [84]^{I}
0.10 to ≤0.20	[85]^{I}, [86]^{C}, [87]^{C}, [88]^{I}, [89]^{I}
0.20 to ≤0.30	[90]^{T}, [91]^{T}, [92]^{T}, [93]^{T}, [94]^{I}, [95]^{I}, [96]^{I}, [97]^{I}
0.30 to ≤0.40	[98]^{T}
Age at puberty	
<0.10	[99]
0.10 to ≤0.20	[49]^{T}, [82]^{T}, [100]^{T}
0.40 to ≤0.50	[101]^{T}, [102]^{T}, [103]^{T}, [104]^{TI}, [105]^{I}, [106]^{I}
≥0.50	[107]^{T}, [108]^{T}, [109], [110]^{CI}, [111]^{C}, [112]^{C}, [113]^{CI}, [105]^{C}, [106]^{I}
Calving date	
<0.10	[90]^{T}, [93]^{T}, [114]^{T}, [115]^{T}, [116]^{T}
0.10 to ≤0.20	[90]^{T}
0.20 to ≤0.30	[91]^{T}, [108]^{T}, [114]^{T}
0.40 to ≤0.50	[117]^{T}
Calving rate	
<0.10	[118]^{T}
0.10 to ≤0.20	[118]^{C}
Calving success	
<0.05	[118]^{T}
0.05 to ≤0.10	[118]^{TC}
Calving to first insemination	
<0.10	[119]^{T}, [120]^{T}, [121]^{T}
Days to calving	
<0.10	[119]^{T}, [118]^{TC}, [121]^{T}, [122]^{T}
First-service conception rate	
<0.10	[123]^{T}
0.10 to ≤0.20	[87]^{C}
0.20 to ≤0.30	[124]^{T}
Heifer pregnancy	
<0.20	[81]^{T}, [88]^{I}, [125]^{T}, [126]^{C}, [127]^{T}
0.20 to ≤0.30	[128]^{T}
0.40 to ≤0.50	[85]^{I}, [129]^{I}, [130]^{I}, [89]^{I}
>0.50	[46]^{I}
Number of calves	
<0.10	[118]^{T}, [131]^{T}
0.10 to ≤0.20	[131]^{T}, [132]^{T}
0.20 to ≤0.30	[133]^{I}
0.30 to ≤0.40	[118]^{C}
Pregnancy rate	
<0.10	[45]^{T}, [93]^{T}, [116]^{T}, [122]^{T}, [134]^{T}
0.10 to ≤0.20	[49]^{T}, [81]^{T}, [123]^{T}, [93]^{T}, [133]^{I}
0.20 to ≤0.30	[81]^{T}, [93]^{T}, [135]^{T}, [136]^{T}
0.30–0.40	[137]^{CI}
Probability of pregnancy	
<0.10	[137]^{CI}
0.10 to ≤0.20	[81]^{T}
0.20 to ≤0.30	[93]^{T}, [135]^{T}, [138]^{T}
0.50 to ≤0.60	[78]^{I}
Scrotal circumference	
0.20 to ≤0.40	[85]^{I}, [96]^{I}, [97]^{I}, [103]^{TC}, [125]^{T}, [139]^{T}, [140]^{T}, [141]^{I}
0.40 to ≤0.50	[43]^{T}, [46]^{I}, [49]^{T}, [54]^{C}, [82]^{T}, [84]^{I}, [85]^{I}, [89]^{I}, [92]^{T}, [93]^{T}, [96]^{I}, [129]^{I}, [130]^{I}, [141]^{I}, [142]^{T}, [143]^{T}, [144]^{T}, [145]^{I}, [146]^{I}, [147]^{I}, [148]^{T}, [149]^{I}
0.50 to ≤0.80	[54]^{I}, [81]^{T}, [85]^{I}, [94]^{I}, [102]^{T}, [112]^{C}, [133]^{I}, [137]^{CI}, [145]^{I}, [146]^{I}, [150]

Source: Adapted from [151].
T, I, and C superscripts represent estimates derived in taurine, indicine, and taurine × indicine crosses, respectively [151], and numbers below the superscript represent the paper from which heritability estimates are derived.

Table 76.3 Table indicating which breeds utilize the various fertility traits used in beef breed evaluations.

	SC	SCF/Stay	HP	30-MP	GL
Angus	X		X		
Charolais	X				
Chianina		X			
Gelbvieh		X	X	X	
Hereford	X	X			
Limousin	X	X			X
Maine-Anjou					
Red Angus		X	X		
Shorthorn		X			
Simmental		X			

Sources: [79, 152–161].
SC, Scrotal circumference; SCF/Stay, sustained cow fertility and stayability; HP, heifer pregnancy; 30-MP, 30-month pregnancy rate; GL, gestation length.

Age at Puberty

Age at puberty is a measure of the age when a heifer first cycles or presents her first corpus luteum that can be palpated. Age at puberty is closely related to HP, as some of the variation in HP can be attributable to breeding failure when heifers are still maturing. Selection for age at puberty allows producers to breed replacement females at an earlier age. Heritability estimates vary extensively and estimates for taurine breeds are lower than indicine or taurine and indicine crosses (Table 76.2).

Pregnancy Traits

Pregnancy and Conception

Heifer pregnancy is a trait currently used in Angus, Gelbvieh, and Red Angus evaluations (Table 76.3) to indicate fertility in replacement females. HP and other pregnancy success traits are binary records (encoded as success or failure) and represent the probability progeny will be pregnant at the end of the breeding season. HP is a direct measure of the likelihood of heifer progeny becoming pregnant as first-calf heifers [79, 151]. Like age at puberty, HP heritabilities tend to vary significantly between taurine and indicine cattle (Table 76.2). Like HP, DPR, cow conception rate, and heifer conception rate are binary traits. DPR is evaluated for all breeds evaluated by the Council for Dairy Cattle Breeding (CDCB) [78] and indicates the likelihood a sire's daughters will become pregnant during a given estrus. Heifer and cow conception rate are analogous to HP in dairy cattle, with the exception of cow conception rate being measured on females that have already achieved first parity. Heifer and cow conception rate are evaluated for all breeds reported by the CDCB [78].

Gestation Length

Gestation length is expressed in days and is measured as the interval from conception to parturition. Gestation length is evaluated in all breeds evaluated by the CDCB as well as in Limousin [78, 154] (Table 76.3). Heritability estimates ranged from 0.27 to 0.64, but tended to be highly heritable and range from 0.40 to 0.50 [164–171] (Table 76.2).

Thirty-Month Pregnancy

Thirty-month pregnancy is a Gelbvieh-specific evaluation [155] (Table 76.3). It is similar to HP but is the probability a cow will calve as a three-year-old if she calved as a two-year-old. It aims to capture variation associated with achieving and maintaining pregnancy as a cow rather than a heifer.

Pregnancy Rate

Pregnancy rate (PR) is a reflection of a female's lifetime productivity and is calculated as the ratio between the number of pregnancies and number of breeding seasons in which she had an opportunity to conceive. PR can be influenced by the length of the breeding season as well as sampling error when a female is exposed to a shorter number of breeding seasons (which may be alleviated by requiring multiple breeding seasons of data) [151]. Furthermore, PR fails to capture variation associated with successfully producing a live calf and only emphasizes a female becoming pregnant at any time, rather than becoming pregnant early on in a breeding season [123] (unlike first-service conception rate, for example). PR tends to be low to moderately heritable (Table 76.2).

Probability of Pregnancy

Probability of pregnancy is the likelihood a female will conceive and remain pregnant. It is binary and the interpretation when comparing two animals is similar to heifer PR and PR. Like PR, probability of pregnancy fails to take into account when an animal becomes pregnant in the breeding season (females conceiving late in the season are treated identically to those that conceive early). Unlike PR, probability of pregnancy does allow for selection on pregnancy maintenance [151]. Interestingly, probability of pregnancy heritability estimates tend to be moderate, but there are few estimates available (Table 76.2).

Calving Traits

Age at First Calving

Age at first calving is an easily measurable trait because it can be calculated using a calf's birth date and the dam's birth date. Selection for age at first calving would reduce the gestational period and select for earlier maturing females [90]. Age at first calving is also favorably correlated with calving interval, calving date, lifetime productivity, and calving age in subsequent calving seasons [91, 151], creating favorable correlated responses to selection. This is intuitive, as heifers calving later tend to have a longer calving interval, a later calving date, and potentially less offspring over their lifetime. However, the date of birth for a given animal greatly influences measurements of age at first calving as animals born later in a calving season tend to be younger when the breeding season occurs. Therefore, selection for age at first calving can lead to indirect selection for heifers born later in a calving season, given breeding seasons tend to be fixed in the beef industry rather than being based on the age of the

animal [90]. Age at first calving heritability estimates range from low to moderately heritable and have been the focus of a large number of studies (Table 76.2).

Stillbirth

Stillbirth is evaluated in Holsteins by the CDCB [78]. Stillbirth indicates the percentage of offspring born dead or that die during the first 48 hours post-parturition. The CDCB estimated the heritability of stillbirth to be 0.03 and 0.065 for sire and daughter stillbirth, respectively [172].

Calving Date and Days to Calving

Calving date is the day of a calving season in which a female calved. For example, when the first female calves, the day she calved becomes day one of the calving season, and an animal calving a week later would have a calving date value of eight. Like age at first calving, it is easily calculated from calf birth date and captures variation associated with early conception in the breeding season and gestation length. Calving date could easily be incorporated into genetic evaluations and avoids some of the unwanted side effects present in selecting for age at first calving, such as selecting for later-calving females [90]. Days to calving is similar to calving date and is measured as the time period from the first AI or bull exposure to parturition. Like calving date, selection for days to calving would select for greater conception rates and shorter gestation lengths. Days to calving is lowly heritable (Table 76.2).

Calving Success

Calving success is a binary trait similar to HP. Unlike HP, calving success can capture variation associated with multiple pregnancies and maintaining a pregnancy through parturition. Calving success has not been widely used and appears to have a very low heritability (Table 76.2).

Calving to First Insemination

Calving to first insemination is a trait used in all dairy breeds evaluated by the CDCB [78]. In the dairy industry, calving to first insemination is measured in days as an interval from calving to the first breeding day [119]. In evaluations conducted by the CDCB, calving to first insemination is estimated as a linear function of DPR and cow conception rate; values are expressed in days, where a larger number indicates a longer interval before a cow begins to cycle again after parturition. Heritability estimates in beef populations are low (Table 76.2).

Lifetime Productivity and Longevity

Productive Life

Productive life is a trait used in all CDCB genetic evaluations [78]. Productive life measures the amount of time in months a female remains in lactation, thus capturing variation associated with producing calves and longevity. It is currently analyzed in a multitrait model with stillbirth, DPR, yield traits, mastitis resistance, type traits, and calving ease traits, which serve as correlated indicator traits to give information about productive life, given they tend to be measured earlier [78]. Heritability for productive life was estimated at 0.08 [173].

Sustained Cow Fertility and Stayability

Sustained cow fertility is a Hereford-specific evaluation similar to stayability [152] (Table 76.3). It is expressed as a probability, where the difference between two animals indicates the probability female progeny will have one more calf by age 12 relative to the second animal. A difference of 10 indicates a female has a 10% greater probability of having one more calf. Sustained cow fertility has gradually been rising in Herefords (Figure 76.12).

Stayability is a more general form of sustained cow fertility utilized in Chianina, Gelbvieh, Limousin, Red Angus, Shorthorn, and Simmental evaluations [154–159] (Table 76.3). Stayability represents the probability progeny of a given animal will remain in the herd till at least six years of age. Stayability captures variation associated with fertility, as animals failing to achieve pregnancy will likely be culled from the herd, but can be biased by other forms of culling (such as culling for faster genetic progress) if culling codes are not used appropriately. Stayability heritabilities tended to be similar across taurine breeds, but increase slightly on average when the age of evaluation increases from three to six years (0.16–0.18) [174].

Calving Rate

Calving rate is the proportion of calves produced by a cow relative to the total number she could have potentially produced. Calving rate is a measure of reproductive efficiency and attempts to capture the entire lifecycle of fertility in a female [118] rather than just achieving pregnancy. However, record collection for calving rate is slow and records are incomplete until late in the productive life of a female. Heritability for calving rate is low but has not been widely studied (Table 76.2).

Number of Calves

Number of calves aims to measure a female's lifetime productivity by measuring the total number of calves over a female's lifetime. Due to the nature of total lifetime productivity traits, data collection occurs later in life, leading to low accuracies until many progeny and grand-progeny have been recorded. This is a general disadvantage, but it underscores the potential for genomic technologies to increase accuracy of evaluation earlier in an animal's lifetime. Number of calves captures information associated with nearly every aspect of fertility (including stayability). Heritability estimates are moderately high in some studies but generally tend to be low (Table 76.2).

Cumulative Productivity

Similar to stayability and number of calves, cumulative productivity, a relatively new trait in the literature, tracks the production over a female's lifetime. Unlike number of calves and stayability, cumulative productivity can be used to ascertain fertility variation associated with stayability and number of calves as well as production (i.e. total weaning weight of all calves rather than just the number of weaned calves). Like stayability, estimates of heritability for different measures of cumulative productivity increase with age. From two years to eight years of age, heritability increased from 0.10 to 0.24 for calves born and 0.04 to 0.23 for calves weaned. Likewise, weight weaned increased from less than 0.10 to 0.36 at eight years [175].

Unlike many male fertility traits, most female fertility traits are considered lowly heritable and data collection is often more difficult. Under these constraints, genetic progress tends to be slow, though it can be made and should not be ignored. Greater immediate gain can often be realized by managing non-additive effects (like heterosis) using crossbreeding systems. Given the importance associated with accurately selecting replacement females due to the substantial economic investment made by producers, genomics can provide much-needed information to bolster genetic prediction for fertility traits.

Emerging Genomic Technologies

The incorporation of genotype data into genetic evaluations is crucial for genetic improvement in the livestock industry. However, there are also many emerging genomic technologies that have the potential for commercialization and utilization within the beef and dairy industries. We will review a number of these technologies in this section – embryonic lethal haplotypes, embryo biopsying, and gene editing.

Embryonic Lethal Haplotypes

A haplotype is a series of SNPs located in close proximity that are inherited together. They can be thought of as blocks of the genome, represented by concatenated SNPs that are inherited together (Figure 76.13). Researchers can use haplotypes to better model the potential causal mutation(s) associated with a trait when the causal mutation is not known.

One type of mutation that is of particular interest to producers is embryonic lethal mutations. These mutations are typically recessive "broken genes" that manifest themselves during embryonic development at the time point when that gene product is needed for development. At this time, spontaneous abortion occurs due to the lack of a necessary gene product and the cow will come back into heat. This will result in greater numbers of open cows (especially with shorter breeding seasons) and cows conceiving much later in the breeding season. The manifestation of these mutations as open cows and the lack of stillborn or abnormal progeny makes these types of mutations particularly hard to identify and manage, absent genomic testing, because their effects cannot be easily separated from other environmental effects on PR. However, these mutations have a unique feature that facilitates their identification: the fact that we never see these alleles/haplotypes in homozygous form in populations of live animals because homozygous animals are never born [176].

There are currently 14 known haplotypes connected to female infertility or progeny loss in dairy cattle, and

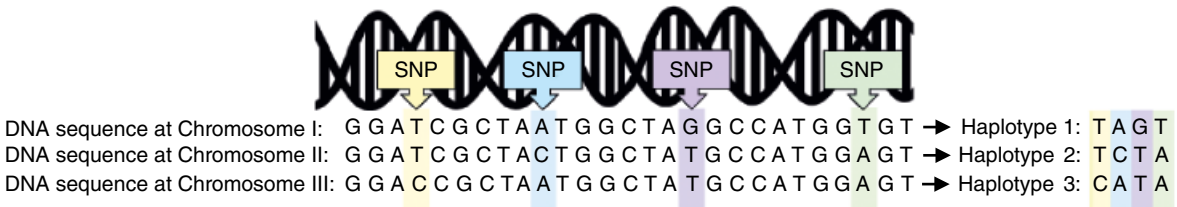


Figure 76.13 Haplotypes are made up of concatenating SNPs.

another 10 fertility haplotypes have been discovered in beef cattle. All 24 haplotypes are summarized in Table 76.4.

Fertility haplotypes that occur at higher frequencies are associated with greater pregnancy loss. For example, in Table 76.4, AA14H3 is present in Angus populations at a frequency of 15.2%, which is higher than any other haplotype listed. This indicates that AA14H3 is more likely to be homozygous in an embryo by chance, thus causing pregnancy loss. In contrast, homozygous haplotype deficiency (HHD) is very rare in Holsteins and is only reported to occur at a frequency of 0.01%, so it is unlikely that two animals chosen at random will both be carriers of this mutation and little pregnancy loss is expected due to this mutation. Fortunately, with genomic testing, these mutations can be managed within the population similarly to the recessive genetic abnormalities already discussed at the beginning of this chapter. As long as two carriers of the same mutation/haplotype are not mated together, there should be no pregnancy loss expected as a result of that mutation. Because of this, producers should not immediately cull heterozygote carriers of a lethal allele, but rather manage the herd and matings accordingly.

Embryo Biopsying and Genotyping

Genotyping of embryo biopsies enables breeders to make selection decisions on animals that have not been born yet. For example, calves with poor genetic merit that would be traditionally culled after birth or those of an undesirable sex can be culled before the embryos are implanted. Embryo biopsying and genotyping are tools that are not yet commonly utilized in cattle but would enable screening of embryos for heritable diseases, genetic defects, and sex in addition to evaluation of genetic merit for production traits before the animal is even born.

Table 76.4 Fertility haplotypes in dairy and beef cattle.

Breed	Haplotype	Chromosome	Region (bp)	Frequency (%)
Brown Swiss	BH1 [176]	7	42 811 272–47 002 161	6.67
	BH2 [177]	19	11 063 520	7.78
Holstein	HCD [178]	11	77 958 995	2.5
	HH0 [179, 180]	21	21 184 869–21 188 198	2.76
	HH1 [161]	5	63 150 400	1.92
	HH2 [176, 181]	1	94 860 836–96 553 339	1.66
	HH3 [181, 182]	8	95 410 507	2.95
	HH4 [13]	1	1 277 227	0.37
	HH5 [183, 184]	9	93 223 651–93 370 998	2.22
	HHC [185]	3	43 412 427	1.37
	HHD [186]	1	69 757 801	0.01
Jersey	JH1 [187]	15	15 707 169	12.1
Ayrshire	AH1 [188, 189]	17	65 921 497	13
	AH2 [190]	3	51 267 548	9.8
Angus	No name [191]	1	27 786 985–29 095 768	2.3
	No name [191]	4	82 467 969–83 996 686	7.6
	No name [191]	8	62 040 920–63 000 189	2.3
	No name [191]	12	59 989 293–61 258 655	3.2
	No name [191]	15	82 317 986–83 144 172	3.8
	No name [191]	17	46 514 063–47 462 424	4.5
	No name [191]	29	43 043 207–44 243 444	4.4
	AA14H3 [192]	14	8 064 004–8 927 881	15.2
Simmental	SI16H5 [192]	16	51 195 450–53 097 936	10.8
Charolais	CH19H2 [192]	19	48 874 999–48 902 319	14.4

The capability to biopsy and genotype animal embryos facilitates quicker generational intervals when making selection decisions for even faster genetic gains. Moreover, biopsying embryos early in development takes advantage of the embryo's natural plasticity in its resilience to environmental stressors [193].

There are many assisted reproductive technologies available to cattle producers, but embryo biopsying is one of the only methods able to guarantee the sex of progeny before birth. Scrutinizing the sex of embryos might sound trivial but can be very important to the overall profit and productivity of an operation. For instance, the goal of many dairy farms is to raise replacement heifers, which can be impaired by a surplus of male births. Although sexed semen technology exists, it can only dramatically increase the chance of producing a calf of the desired sex. Fetuses can be sexed by ultrasound beginning 60 days after conception [194], but biopsied embryos allow filtering of embryos based on sex before implantation occurs.

Furthermore, genotyping embryos can reveal the alleles present for deleterious genetic conditions or important SNPs of interest [1]. With the increased use of genomic testing, it has become more common to find livestock producers not only genotyping animals at birth, but also now genotyping embryos [1]. Another advantage to genotyping embryos is the ability to reduce the costs associated with multiple embryo transfers by selecting only the most desirable embryos for implantation [195].

There are two approaches to cultivating embryos for biopsying and genotyping: *in vivo* (VV) and *in vitro*-produced (IVP). VV embryos are cultivated in the living animal and flushed for genomic evaluation, whereas IVP embryos are grown in laboratory settings, with oocytes collected from the dam [196].

Once the fertilized egg reaches the blastocyst or morula stage, it can be biopsied for genomic analysis and subsequently implanted into the recipient dam. Biopsying an embryo requires aspirating part of the trophectoderm, or inner cell mass, and extracting its DNA for further evaluation [193]. However, this requires breaching the protective outer layer of the embryo, the zona pellucida. Thus, a biopsied embryo is compromised and at a greater risk of degradation than an embryo with an intact zona pellucida [193]. If embryos cannot be implanted immediately after biopsy, they must be cryogenically preserved, which can also increase risk of damage to the embryo and influence embryo viability [193]. There are three commonly used approaches to collect embryonic cells for genotype analysis: needle, aspiration, and microblade techniques.

Studies have concluded that there is no significant difference in embryo viability between the three common biopsy techniques when embryos are implanted soon after the procedure [193, 197], but embryos that have been cryogenically preserved have a greater chance of survival when the zona pellucida remains as intact as possible [193].

A study from 2012 compared the effectiveness of each technique and found that frozen embryos biopsied via the needle technique yielded a PR of 57%, but those biopsied with the aspiration and microblade techniques were associated with lower PRs at 43 and 31%, respectively [193]. The greater pregnancy successes of needle and aspiration techniques are attributed to less zona pellucida damage, but these techniques are more laborious and yield less DNA than the microblade method.

In 2016, the cost of embryo biopsy and genotyping was estimated at $150 USD, which is more expensive than many other assisted reproductive technologies [198]. Although embryo biopsies involve similar levels of risk to the oocyte donor and recipient dam as embryo transfers, embryo biopsying is an invasive procedure that presents an additional level of risk for the embryo. The success of implanting a biopsied embryo will ultimately depend on technician skill, the embryo's developmental stage at the time of biopsy, biopsy technique, time between biopsy and implantation, and animal health. Embryo biopsying is a relatively recent genomic tool that has the potential to expedite genetic improvement in the livestock industry, but its practical application and cost-benefit ratio may vary among species and individual producers.

Gene Editing

Genomic selection can increase the productivity and profitability of cattle enterprises, but gene editing, or precision breeding, could be used as an effective complement to genomic prediction in cattle. Gene editing is currently most practical for simple Mendelian traits controlled by one mutation. Quantitative traits involve the interaction of many alleles, and for most traits the causal mutations are unknown, thereby increasing the potential complexity involved in precision breeding. Gene editing is the process of permanently altering the genomic sequence at specific sites in the DNA sequence [199]. There are three major gene editing tools used today: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR). Although these methods all result in permanent genome modification, the molecular mechanisms behind them differ. Even if the mechanisms differ, all of them involve making targeted cuts to the DNA sequence and allowing the cell's repair mechanism to either directly repair the break (often silencing the gene where the cut was made) or repair the break by inserting

donor DNA (which can be used to add a sequence of interest to the genome or "correct" mutations within the DNA).

Once the site-directed nuclease system, either ZFNs, TALENs, or CRISPR, cuts the targeted DNA sequence, scientists use the cell's natural DNA repair machinery to permanently alter the genome. Once both DNA strands are cleaved, the cell will perform one of two repair processes. Both are illustrated in Figure 76.14. The first is non-homologous end joining (NHEJ), whereby the DNA strands are annealed back together. However, this repair process typically causes a mutation (addition or deletion of a DNA base or bases) in the sequence. The mutation(s) introduced by NHEJ often disrupt the gene enough to render it inactive, resulting in what is known as a knockout. Knockouts can be useful in determining the function of a gene and its importance for normal growth and development. They could also be used to prevent a particularly detrimental gene from being expressed. Alternatively, the cell will undergo homology-directed repair (HDR) if a separate strand of intact DNA is used as a template to reconstruct the broken strands. The specificity by which HDR functions is important for introducing a predesigned sequence to replace the nucleotides originally at the locus of interest. This process can be used to correct deleterious alleles associated with heritable diseases or disorders, as an example. Single nucleotides can be pinpointed and altered, or entire strings of nucleotides can be added to the sequence of interest by taking advantage of the cell's natural HDR mechanism [200].

In the cattle industry, applications of gene editing technology have mostly centered on traits with large phenotypic effect as a proof of concept. For example, some mutations in the myostatin gene (*MSTN*) result in the double-muscled phenotype often seen in Belgian Blue and Piedmontese cattle. In 2015, researchers out of Texas A&M University altered the *MSTN* gene in a Nelore bull so that it would be identical to the mutation found in Belgian Blue cattle responsible for at least part of the double-muscled phenotype. This simple edit resulted in a Nelore bull with increased muscle mass and increased retail product [201].

Gene editing technology has also shown potential in animal management. For example, polled cattle present a lower risk of injury to themselves, their handlers, and their herdmates than their horned counterparts [202]. Dairy cattle tend to be more intensively managed than beef cattle, making polled animals even more valuable in dairy operations. However, the POLLED allele is currently much more common in beef animals than dairy. Traditional selection for the polled trait in dairy cattle would be slow and the necessary backcrossing to either polled beef cattle or polled dairy cattle (which have substantially lower average milk production than horned dairy cattle) would result in a dramatic decrease in performance. Gene editing offers a way to fast-track this process by inserting the polled allele into the genome of an animal with exceptional milk production performance [203]. It is far easier to incorporate polled alleles into dairy cattle embryos through precise gene editing rather than traditional breeding methods [204].

Additionally, gene editing could be used to correct deleterious alleles, like lethal recessives. Recent research in mice has demonstrated that prenatal gene editing can alter nucleotides responsible for producing lethal phenotypes to increase survivability after birth and mitigate developmental abnormalities [205, 206]. This has the potential to easily and effectively eradicate recessive lethal alleles from the population without any negative effect on cattle performance, such as we might observe if carriers are culled indiscriminately from the population.

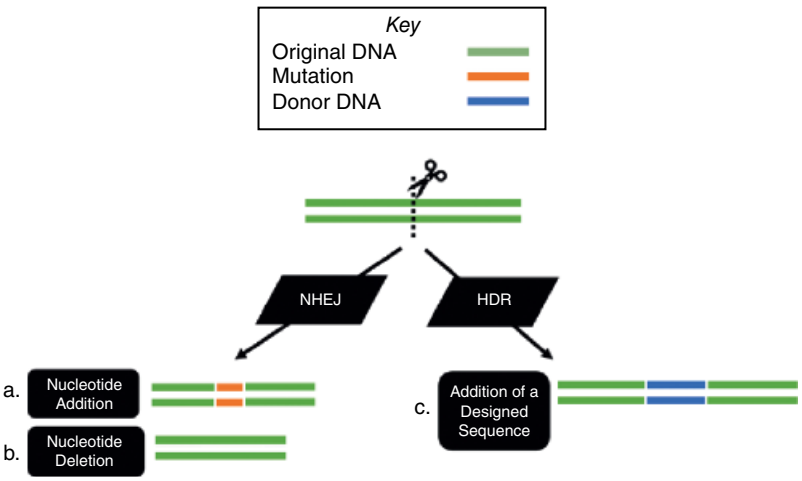


Figure 76.14 DNA repair processes.

Summary

Genomic technologies have enabled producers to more effectively select the most desirable animals, manage genetic abnormalities, improve management, and make rapid genetic progress. New genomic tools, such as embryo biopsying and precision breeding, hold promise that breeders will soon be able to directly and precisely influence the genetic merit and characteristics of their herds to better fit various breeding objectives [207]. As genomic technology continues to advance, there is no doubt that the livestock industry will benefit greatly from the genomic developments ahead.

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Technological Advances that Enhance the Quantity and Quality of Spermatogenic Production

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Feeding the 10 Billion Human Population

The human population will grow to about 10 billion by 2050 [1, 2]. Feeding the 10 billion will require enhanced livestock production which necessitates improved livestock reproductive efficiency. While we can control some other inputs for more efficient food production, the genetic potential of animals is an intrinsic limiting factor which cannot be controlled after birth. Thus, there is a need to further enhance the genetic potential to boost more efficient food production. The extensive use of elite gametes is the best tool to enhance this [3] by increasing genetic potential. Human beings have been breeding animal females selectively with elite males since the domestication of animals. Artificial insemination (AI) has been used to spread elite male germplasm especially in dairy production since the 1960s [3]. As the male germplasm is the major tool to spread elite genetics, spermatozoa are and will remain the main target to fill the need [3]. Both the quantity and quality in terms of genetic potential of spermatozoa from the bull must be improved to ensure profitable and efficient spread of elite genetics for more efficient food production. The increased quantity of spermatozoa per animal will decrease the price of the semen, while the increased genetic quality will enhance the production potential of the progeny.

Increasing the Quantity of Spermatogenesis per Bull

Historically and traditionally, males of livestock species have been selected for breeding based upon scrotal size. Increased sperm production from the elite male will enhance the speed of genetic improvement in livestock through widespread use of desired genetics. Increased sperm production per bull will decrease the cost of semen production per bull at a fixed cost of maintenance. The bulls will produce more spermatozoa per ejaculate, enhancing their utilization in semen sexing programs. Sexed semen enables rapid herd expansion by supplying replacement heifers for dairy and increases profit through male calves for beef production [4]. One of the criterion for a bull to be enrolled in a sex sorting program is that it must give ejaculate(s) with six billion spermatozoa for each run of the semen sexing process [4]. Lower daily sperm production (DSP) in some adult bulls makes them unsuitable for supplying these many spermatozoa for the semen sexing process, hence such bulls are not enrolled to sex sorting programs even if they possess elite production potential. Moreover, younger bulls cannot produce the required six billion sperm cells for each run of the semen sexing process because they have lesser DSP. So, such bulls are enrolled in sex sorting programs at or after two years of age. As the bull attains puberty at about one year of age, this late enrollment at or after two years of age delays genetic improvement [4]. An increase in DSP will reduce the problem of no enrollment to the semen sexing program of a bull with lower DSP or late enrollment due to lower DSP in younger bulls at or after two years of age. Thus, an increased DSP in the bull will enhance the speed of genetic improvement through sex sorting [5]. Moreover, the cost of bull maintenance and DSP are major factors affecting the cost of sexed semen and of AI [6, 7]. The cost of semen production can be reduced by obtaining more spermatozoa per bull at the fixed cost of maintenance per bull, i.e. by increasing DSP per bull. Increased DSP in the bull is advantageous for rapid genetic improvement in cattle production, for decreasing cost of semen production in (AI) and semen sexing programs, and for obtaining more spermatogonial

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

stem cells (SSCs) for SSC transplantation [8], and therefore is of interest to livestock industries.

Methods to Increase Sperm Production per Bull

Several methods including unilateral castration, immunization against inhibin, and immunization against estradiol have been used to increase the testicular size and DSP of the bull; however, none of these has gained commercial success [8, 9]. DSP in the bull is positively correlated (R = +0.83) to the number of total Sertoli cells (SCs) per testes and becomes fixed once proliferation of SCs stops [10]. SC number in the testes of an individual, excluding some seasonal breeders, is fixed at a species-specific age when SCs have differentiated and stop dividing indefinitely. A potential method to increase DSP in the bull is by increasing SC number per testis through the induction of temporary hypothyroidism [5].

Thyroid Hormone and Testicular Function

Effect of thyroid hormone (TH) on body organs including liver, kidney, and skeletal muscles is by indirect actions on the tissue metabolism [11]. However, TH alterations do not affect testicular metabolism [11] or testicular function [12] in the adult animal. Unlike other body organs, adult testes do not respond metabolically to TH by increasing oxygen consumption and mitochondrial alpha-glycerophosphate dehydrogenase and possess meager TH receptors [13]. Thus, adult testes are not affected by hypothyroidism or hyperthyroidism. Conversely, adverse effect of TH manipulation, precisely of hypothyroidism, on prepubertal ovarian [14] or testicular [15] function has been known for a long time. Maqsood (1952) [15] reported that hypothyroidism inhibited testicular development and delayed the onset of spermatogenesis. Though these effects of hypothyroidism on testicular function could have been attributed to effects of the deficiency on testicular metabolism or gonadotropins, effects of TH on the testicular function were verified as direct actions of TH on testicular parenchyma during the late 1980s [8].

Though adult testes lack significant TH receptors, demonstration of TH receptors in neonatal testes suggested a possible role of TH in testicular function during prepubertal development [8]. Nuclear receptors of thyroid hormone (TNRs) [16] and associated mRNA [17] are highly expressed in neonatal testes predominantly in SCs and the expression is the highest until a few days after birth in the rat.

Postnatal hypothyroidism inhibits SC maturation [18], delays seminiferous tubular lumen formation by inhibiting proluminal fluid secretion from SCs and increases germ cell degeneration [19]. TH also affects secretion of androgen binding proteins [20] and insulin-like growth factor (IGF)-1 [21], glucose transport [22], protein synthesis and other metabolic processes of developing SCs [18].

Thus, TH is required for proliferation, capacity building to support complete spermatogenesis, development of secretory function, and differentiation and maturation of SCs [8].

Induced Transient Hypothyroidism (ITH)

Hypothyroidism adversely affects SC development and hence testicular function, and thyroxine treatment reverses these adverse effects [23]. *However, if hypothyroidism is induced transiently, the animal reverts to euthyroidism and the induction helps to boost sperm production through increasing SC number* [5].

ITH Enhances Sperm Production in the Bull

To increase sperm production transient hypothyroidism must be induced before the time-point when SC division stops and must be stopped sometime after the time-point. The bull must return to euthyroidism after the induction is removed, otherwise permanent hypothyroidism will adversely affect testicular function. SC proliferation in the bull stops sometime between 4.5 and 5 months of age [5]. ITH was successfully achieved in hybrid beef bulls (Angus cross Wagyu) with the antithyroid compound methimazole (2 mg/kg body weight) twice daily from four to six months of age [5]. Based on serum levels of triiodothyronine (T_3) and thyroxine (T_4), induction of a hypothyroidic state occurred by three weeks of treatment, before the bulls had reached five months of age. Following the treatment period, serum levels of T_3 and T_4 returned to normal by about 6.25–6.75 months of age of the bulls.

During the age period when puberty is normally established in the bull (10–16 months), mean body weight and scrotal circumference were significantly reduced for the treated bulls compared to the control group. However, by 16 months of age both parameters were not significantly different between the two groups. These findings indicate that transient induction of hypothyroidism during the period of SC development delays the normal timeline for the onset of puberty in bulls. Although the growth rate is reduced during the peripubertal period, ITH does not affect mature body weight. ITH resulted in ~2.3-fold increase in SC number and increased sperm production from the treated bulls. The treated bulls produced ~30–180% (i.e. 1.3- to 2.8-fold) more spermatozoa compared to the control bulls at 24–28 months of age. The fresh sperm motility or

morphology and the post-thaw survivability and motility were not affected by the temporary hypothyroidism. Cleavage and blastocyst rate for the freshly collected or post-thaw spermatozoa after *in vitro* fertilization (IVF) were not different between the groups. The treated bulls had a 22% increase in paired testes weight and 50% increase in paired epididymal weight, reflecting increased testicular and epididymal germ reservoir. Thus, transient induction of hypothyroidism during prepubertal development in bulls leads to permanent increase in normal spermatogenesis in adulthood [5].

ITH extends the period of SC proliferation by slowing down the inhibition of their proliferation. During the treatment phase, ITH results in germ cell degeneration, delay in seminiferous tubular lumen formation, and smaller diameter of the seminiferous tubules. However, once the treatment is stopped, these maturational defects are quickly reversed and normal testicular development and maturation starts [24]. ITH increases number reduces volume of LH receptors and decreases steroidogenic capacity of Leydig cells. However, their increased number compensates for the reductions to maintain normal blood testosterone levels [25]. The absence of TNRs from Leydig cells suggests that their number increases in response to paracrine factors from an increased number of germ cells, peritubular myoid cells, and particularly SCs [8].

Possible Mechanism of ITH

Two hypotheses have been proposed regarding the mechanism of action of TH on SCs.

1) TH acts on SCs through cell cycle proteins: cyclin-dependent kinase inhibitors (CDKIs) $p27^{Kip1}$ and $p21^{Cip1}$. TH upregulates $p27^{Kip1}$ (CDKN1B) and $p21^{Cip1}$ (CDKN1A) which then inhibit subsequent phosphorylation activity of cyclins D and E. In proliferating SCs, cyclins D and E bind to cyclin-dependent kinases (Cdk) 4 or 6 and Cdk 2. Once the cyclin–Cdk complex is activated, it phosphorylates the pocket proteins, p^{Rb}, p^{130}, and p^{107}. This phosphorylation releases transcription factors of the E2F family which activate downstream genes for progression of the cell through G1 restriction point [26]. This mechanism has been suggested by the findings that TH induces the expression of $p27^{Kip1}$ and $p21^{Cip1}$ in neonatal murine SCs, whereas ITH decreases $p27^{Kip}$ [27, 28]. Moreover, $p27^{Kip1}$ knockout (p27KO), $p21^{Cip1}$ KO (p21KO), and p27/p21 double-KO (DBKO) mice showed 42, 27, and 86% increase in testes weight, respectively. SC numbers were increased by 126, 48, and 126%, respectively. DSP was also increased in all the knockouts. However, p27KO had 5% and DBKO had 1.5% seminiferous tubules with abnormal spermatogenesis as compared to only 0.5% for the wild-type control. Furthermore, in KO mice, developing germ cells, pyknotic spermatogenic cells, and fewer spermatozoa were found within the epididymides [26].
2) The finding that hypothyroidic treatment of p27KO and DBKO mice results in increased testes weight and DSP [29] suggested that ITH actions on SCs involve some other mechanism also. Another possible mechanism involves Connexin43 (Cx43). Just before puberty, SCs form specific intercellular junctions with neighboring SCs and with adjacent germ cells [30]. Connexin-based gap junctions are cell membrane channels that allow intercellular communication and thus control cell proliferation and differentiation [31]. Cx43 is the most abundant testicular gap junction protein [32]. Inhibitory effect of TH on SC proliferation is associated with a time- and dose-dependent increase in Cx43 which increases gap junction communication and stops SC proliferation. Oleamide and glycyrrhetinic acid, specific blockers of gap junction coupling, reverse the inhibitory effect of TH on SC proliferation [33]. SC-specific Cx43 knockout (SC-Cx43 KO) mice are infertile and have continued SC proliferation, actively proliferating early spermatogonia without advancement to subsequent spermatogenesis and delayed age at maturity [34–36].

Transgenesis to Increase Sertoli Cell Number

Application of CRISPR/Cas technology to livestock may be used to increase SC number and DSP. Knocking out $p27^{Kip1}$, $p21^{Cip1}$, or Cx43 may lead to an increase in SC number and DSP as seen in the mice. However, the knockout mice had abnormal spermatogenesis and were infertile. At present, induction of transient hypothyroidism to increase SC number is the most applicable method to increase DSP in the bull. Moreover, ITH to increase SC number in germ cell ablated *NANOS2*-knockout surrogate bulls may increase the success rate of SSCs transplantation in the bull.

Improving the Genetic Quality of Spermatogenesis

The majority of genetic improvement in cattle production has resulted from expansion of elite male germplasm through AI. In the USA, adaptation of AI led to a 4.5-fold increase in milk production per average dairy cow from 1940 to 2009 [37]. While AI has been a successful tool for genetic gain in dairy cattle, it has not been adopted by beef

cattlemen due to different management systems. Only about 5–10% of beef cattle production systems use AI, in contrast to 80% of dairy production systems. Thus, the male germplasm from elite bulls cannot be spread through AI in beef production systems. Through natural mating, an elite bull can impregnate only a limited number of cows per breeding season based upon its DSP. Hence, there is a need to find a suitable technique to spread the spermatozoa from elite beef bulls to large-scale beef production. One such possibility is through donor-derived spermatogenesis in surrogate sires [3].

Surrogate Sires

A surrogate sire refers to a bull that lacks his own germ cells but produces spermatozoa of another bull after SSC transplantation [38] (Figure 77.1). Having many recipient surrogate bulls producing donor-derived spermatogenesis of desired elite genetics will preserve the elite genetics for indefinite time, hasten the spread of desired male genetics [37, 39], provide more bulls producing genetically elite spermatozoa for natural mating in beef production, and decrease cost of semen production in AI programs. Additionally, spermatozoa of an elite prepubertal bull calf can be produced in a recipient by transplantation well before the donor would have reached puberty [3]. Importantly, this will solve the problem of poor semen quality of AI bulls of temperate breeds, imported to tropical conditions [40–42], as recipient bulls local to the tropical regions can produce spermatozoa of the elite temperate breeds not well adapted to the tropics. Such donor-derived spermatogenesis in the recipient bull from the donor bull requires: (i) ablation of the germline of the recipient so that it does not produce its own spermatozoa but has normal libido, physiology, and testicular architecture; and (ii) transplantation of germ cells/SSCs from the testes of an elite bull to the testes of the donor bull. The objective is the production of elite spermatozoa of the donor haplotype by the germ cell ablated recipient bull [3]. The ultimate success in such transplantation is fertile donor-derived spermatogenesis without any indigenous spermatogenesis so that all (100%) next generation will be from the spermatozoa of the elite donor genetics [3].

Recipient Preparation: Ablation of Germ Cells

SSC transplantation leading to commercially adaptable continuous donor-derived spermatogenesis has not been successful in any livestock species. One major limiting factor for success of this technique in cattle has been unavailability of fully germ cell ablated recipients having intact seminiferous epithelium and testicular somatic cells [43]. One fundamental requirement for donor-derived spermatogenesis without any indigenous spermatogenesis is complete ablation of the germ cells in the recipient testes. While the germ cells are ablated, the somatic cells and histological architecture of the testes must not be disturbed. Possible ways to ablate indigenous germ cells are as follows: (i) chemical ablation with

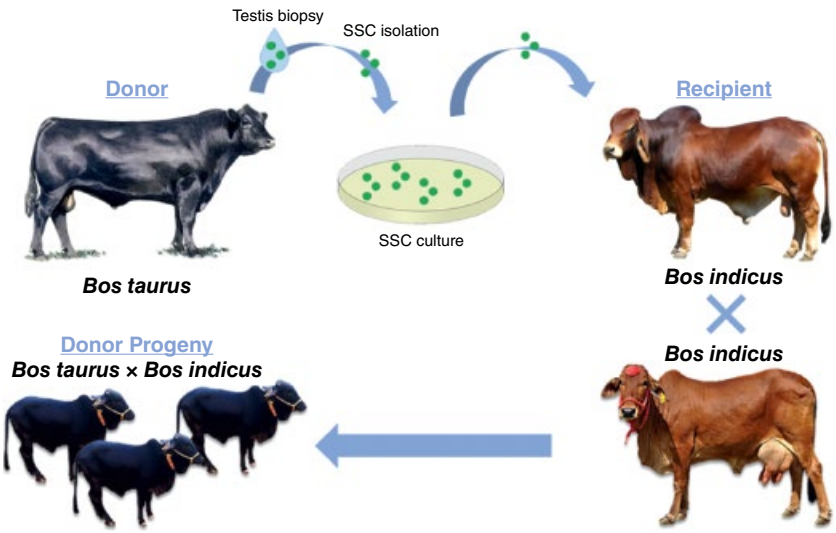


Figure 77.1 Schematic representation of surrogate sire technology. Spermatogonial stem cells (SSCs) can be obtained from an elite sire (*Bos taurus* here) through testicular biopsy. SSCs can be expanded *in vitro* to increase their number before transplantation to a germ cell ablated recipient (*Bos indicus* here). Breeding the recipient *Bos indicus* bull to a *Bos indicus* female will result in *Bos taurus* × *Bos indicus* progeny.

chemotherapeutic agents such as busulfan is practiced in rodents for germ line ablation in research settings. However, the technique is practically difficult for the bull due to the large dose required; (ii) degeneration of germ cells with high-dose testicular X-ray irradiation has been studied in cattle [44]. The ablation and subsequent donor's germ cell transplantation resulted in donor-derived spermatogenesis. However, the indigenous germ cells were not completely degenerated. Moreover, both donor- and recipient-derived spermatozoa were present in the ejaculate. Thus, this technique did not gain commercial success; (iii) a recently studied technique is the ablation of indigenous male germ line through gene editing. Inactivation of the *NANOS2* gene in mice and pigs completely ablates germ cells from testes by apoptosis of prospermatogonia; moreover, histological organization of seminiferous tubules and levels of serum testosterone are not affected [43, 45, 46]. Such genetically modified recipient cattle could serve as suitable recipients for exogenous SSCs transplantation. Embryologically, SSCs are derived from prospermatogonia at species-specific time in the testicular development process [47]. Apoptosis of prospermatogonia is expected to be the method of choice to prepare recipient males for exogenous SSC transplantation in livestock [48]. *NANOS2* is expressed in male primordial germ cells and *NANOS2* knockout mice lack germ cells in intact seminiferous tubules [45, 46]. Recently, the strategy to eliminate prospermatogonia by biallelically inactivating *NANOS2* to completely ablate germ cells has been successful in male pigs. The pig had histologically normal seminiferous tubules and normal serum testosterone levels [43]. Both in pigs and mice, homozygous *NANOS2* knockout (biallelically gene edited) males lack germ cells, while homozygous and heterozygous knockout (monoallelically gene edited) females and heterozygous knockout males are fertile [43, 45, 46]. Practically, biallelically *NANOS2* gene edited males lacking any germ cells will serve as suitable recipients for exogenous SSC transplantation. Furthermore, breeding of biallelically *NANOS2* gene edited females and monoallelically *NANOS2* gene edited males, both fertile, can be used to generate more biallelically knockout males (Park et al., 2016).

Presently, *NANOS2* gene edited males are the best candidates for surrogate sire technology to trasplant elite SSCs and subsequent donor-derived spermatogenesis [3]. However, the production of *NANOS2* gene edited bulls, testing their ability to support donor-derived spermatogenesis without any indigenous spermatogenesis, and the birth of offspring of the elite donor-derived genetics are under study [3].

Germ Cell Transplantation

The germ cells are transplanted through ultrasound-guided needle injection into the rete testis through gravity flow [49]. In the author's experience, the gravity flow of the germ cell suspension solution into the rete testis is more efficient for filling the seminiferous tubules than pushing the cells into the rete testis by using a peristaltic pump. The following cells can be transplanted [50] (Figure 77.2):

- SSCs
- Total testicular digest as it has SSCs
- Non-teratoma-forming subpopulations of primordial germ cell-like cells derived from:
- embryonic stem cells from an embryo of elite genetics
- mesenchymal stromal cells from any tissue
- induced pluripotent cells obtained after inducing pluripotency in somatic cells.

SSCs are considered the most suitable candidate for transplantation into the recipient testes. Spermatogenesis in the seminiferous tubules of testes produces millions of spermatozoa per day. SSCs residing on basement membrane of seminiferous tubules provide more germ cells, along with maintaining their own number, by their divisions for this enormous sperm production per day [47, 51]. SSCs reestablish spermatogenesis after their transplantation to germ cell depleted recipient testes [52]. The donor-derived spermatogenesis can produce offspring of the donor haplotype [53]. Donor-derived spermatogenesis after exogenous SSC transplantation is well established in mice and currently is the main criteria to study functional characteristics of rodent SSCs [54]. Also, donor-derived spermatogenesis after germ cell transplantation has been reported for non-human primates [55], goat [56], pig [57], and cattle [44]. However, offspring from donor-derived spermatogenesis or continuous robust donor-derived spermatogenesis has not been achieved in cattle [37].

SSCs can be obtained from testicular biopsy, expanded *in vitro* through SSCs culture and then transplanted to recipient testes. However, at present, an efficient culture system for bovine SSCs is not available [38].

The surrogate sire technology could materialize into a commercially viable tool for rapidly expanding desirable genetics, thereby having a positive impact on cattle production systems worldwide.

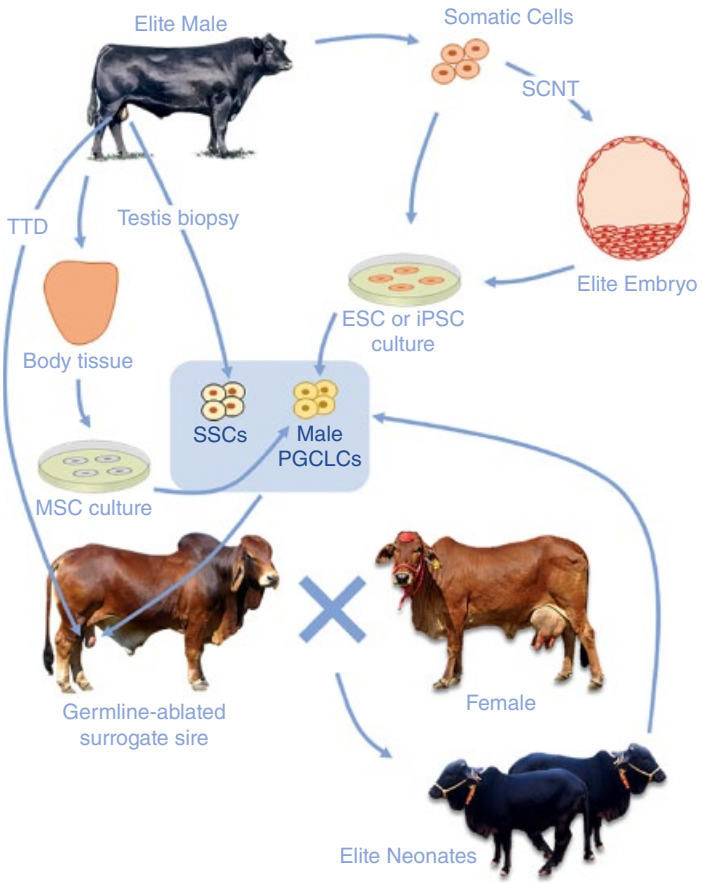


Figure 77.2 Options for donor germ cell types for germ cell transplantation to a germline-ablated recipient bull. Testes can be digested to single cell suspension and total testicular digest (TTD) transplanted to the recipient testes. SSCs obtained from testicular biopsy can be expanded *in vitro* before transplantation to a recipient bull – the most applicable and logical option. Primordial germ cell-like cells (PGCLCs) can be derived from an embryonic stem cell (ESC) of an elite embryo obtained after somatic cell nuclear transfer (SCNT) using elite male genetics, from mesenchymal stem cells (MSCs) from some body tissue of the elite male, or from induced pluripotent stem cells (iPSC) derived from an elite bull. *Source:* Modified from [50].

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Cryopreservation of Semen

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Introduction

Artificial insemination (AI) and cryopreservation of spermatozoa are probably the two great advances that have revolutionized the breeding industry. The dairy industry in particular has benefited from the extensive use of AI, which has permitted an accelerated rate of genetic selection and improvement. It is estimated that more than 60% of dairy cattle in the USA are bred with AI programs as compared with just 10% of beef cattle. The history of semen cryopreservation dates back half a century to the discoveries of the protective agents in egg yolk for cooling and glycerol for freezing fowl and bull sperm [1] and the birth of the first calf by AI using frozen/thawed spermatozoa [2]. Bratton et al. [3] in their field trials demonstrated that bovine sperm frozen to −79 °C and packed on dry ice could still yield high fertility. Since then several media formulations termed "extenders" have been investigated based on their ability to improve the survivability and post-thaw motility of cryopreserved sperm. Similarly, several cryoprotective compounds have been investigated for their protective role during cryopreservation of semen. There is little doubt of the profound effect this technology has had on the cattle industry. This has largely been possible due to the remarkable success that has been achieved with bull semen as compared with semen of other species. This has mainly been due to the higher tolerance of bovine sperm to cryoprotectants such as glycerol as compared with other species and the relatively few number of spermatozoa required for conception [4].

Principles of Cryopreservation

Mechanisms of Cell Injury During Cryopreservation

Semen cryopreservation techniques have been in practice for the past several decades and allow long-term storage of semen and hence its virtually unlimited availability. This facilitates the large-scale provision and dissemination of highly valuable genetic material at will. Cryopreservation of semen involves the freezing of spermatozoa to −196 °C, the boiling point of liquid nitrogen, a commonly used medium for freezing and storage purposes. This increases the viability of the individual cells by slowing down their metabolic rate, thereby reducing the rate at which substrates are used and toxins are produced. Semen may also be stored after cooling to 5–8 °C and will survive for 24–48 hours without a significant decline in motility, and even up to 96 hours without a significant drop in fertilization rates. Although this may provide an efficient and successful means of short-term storage, it has some adverse effects on the spermatozoa manifested as a decline in viability rate, structural integrity, motility, and conception rates [5, 6]. Cryopreservation, on the other hand, offers the option of indefinite storage of semen in liquid nitrogen with acceptable post-thaw fertility rates. Cryopreservation can be detrimental to sperm function and fertility even with the latest advances in techniques. However, it is not the long-term storage of cells at these temperatures that is damaging, but rather the progression to these temperatures

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

and back to normothermia which results in cryoinjury [7]. Thus the changes that occur during freezing are mainly ultrastructural, biochemical, and functional. These can impair sperm transport and survival in the female reproductive tract and reduce fertility in domestic species [8].

The effects of cryopreservation on function and viability have been extensively studied for bovine sperm. Spermatozoa have a very limited biosynthetic activity of their own and depend mostly on catabolic processes to function [9]. Thus to halt these metabolic processes the cells need to be cooled below −130 °C. Sperm, like other cells in the body, are composed of various organelles containing water, which can form intracellular and extracellular ice crystals during the freezing process. At temperatures around −5 °C the intracellular and extracellular water remains unfrozen in a supercooled metastable state. However, between −5 and −10 °C, ice forms in the extracellular medium, while intracellular water remains supercooled. At this point, the rate of cooling must be slow enough to permit cellular dehydration to occur, avoiding the freezing of the intracellular water yet fast enough to avoid exposing the cell to a hyperosmotic condition subsequent to dehydration. Severe dehydration leads to solution-effect injury caused by denaturation of macromolecules and extreme shrinkage of the cell up to irreversible membrane collapse [10]. Another damaging effect is the mechanical stress of ice formation all around the cell, which will be constrained to a very limited space of unfrozen solutes [10]. Cell viability plotted as a function of rate of freezing presents an inverted U-shaped curve. Primary causes for cellular damage are largely dependent on the cooling rate employed during the freezing process. The most appropriate freezing rate is the fastest one that allows freezing of extracellular water without intracellular ice formation. Fast cooling between 30 and 0 °C results in cell injury in some sperm cells, called "cold shock," with deleterious effects on the cytoskeleton and genome-related structures and causing cytoplasmic fracture [11]. Cold shock also alters the permeability of various membrane structures on the plasma membrane, mitochondria, and acrosome. During cryopreservation, ice crystals form in the extracellular medium, increasing the osmolality of the unfrozen water. Because of this difference in the osmotic gradient, the intracellular water diffuses out of the sperm, thus dehydrating the cell and the plasma membrane. At thawing, this phenomenon is repeated in reverse order as extracellular ice crystals melt and water starts diffusing in and rehydrating the cell. Because of such drastic changes in the volume and osmotic stress, there can be irreversible damage and ultrastructural deformation of the plasma membrane.

Sperm membranes are composed of many phospholipids (depending on species), with each phospholipid having a precise phase transition temperature. The degree of structural damage to these membranes depends on the temperature and the lipid composition of the membrane [12]. The characteristics of membranes that affect their sensitivity include cholesterol/phospholipid ratio, content of non-bilayer-preferring lipids, degree of hydrocarbon chain saturation, and protein/phospholipid ratio [1]. Some lipids aggregate in domains of gel-like (frozen) lipid, thus excluding other lipid types that remain in the liquid crystalline (melted) state [1, 13]. This ultimately leads to phase separation in which membrane proteins can become irreversibly clustered, leading to loss of function [14]. The specific phase transition temperatures for the different phospholipids in the membrane result in lateral migration with rearrangement of membrane components and lipid phase separations within the plane of the membrane. The lateral migration may create microdomains of non-bilayer-forming lipids and may modify protein surrounding environments. On thawing, these alterations predispose apposing membranes to fuse and affect protein activity, leading to overall altered membrane permeability to water and solutes [1]. This loss of membrane permeability also interferes with ion pumps and results in influx of Ca^{2+} into the sperm cell as well as loss of membrane ATPase activity. Damage to the Ca^{2+} regulatory systems by freezing and thawing may predispose spermatozoa to inaccurate timing of capacitation and the acrosome reaction, contributing to reduced fertilizing capacity of cryopreserved bull semen [15].

Another side effect observed as a consequence of cryopreservation is the generation of reactive oxygen species (ROS) leading to a reduction of the functional and structural integrity of the spermatozoa [16]. The main ROS that are known to affect sperm functionality are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and most hydroxyl radicals (-OH). During the cryopreservation process, large amounts of cytoplasmic components with antioxidant activity could also irreversibly leak into the intracellular space, making the cell vulnerable to the action of these free radicals [17].

DNA integrity and chromatin structure have been identified as the crucial factors related to ability of the sperm cell to endure the cryopreservation process and support embryonic development [18]. Freeze-thawing adversely affects the DNA integrity of cells, making the DNA vulnerable to molecular and epigenetic modifications which can affect embryo development [19]. DNA damage can occur due to several mechanisms during cryopreservation such as: double strand breaks due to high levels of ROS production [20], impairment of DNA repair enzymes [21], and mechanical stress of genomic regions of the DNA molecule in which chromatin compaction is increased because of cell shrinkage [22]. Apoptosis has been correlated with cryoinjury of

sperm DNA and that excessive generation of ROS can cause DNA damage. These parameters can differ greatly between fertile and subfertile bulls [23].

Role of Extenders Used for Cryopreservation

Extenders or diluents are routinely added to protect sperm during liquid storage or cryopreservation. Regardless of species, the role of semen extenders is to (i) provide nutrients as an energy source; (ii) buffer against harmful changes in pH; (iii) ensure appropriate physiologic osmotic pressure and concentration of electrolytes; (iv) prevent growth of bacteria; (v) protect from cold shock during cooling; and (vi) have cryoprotectant(s) to reduce the amount of freezing damage [24]. Over the past several decades, the cryoprotective media for sperm storage have been continuously revised but the basic ingredients remain unchanged, with egg yolk and/or milk and glycerol representing the indispensable compounds of practically all media used for bull sperm preservation in the liquid or frozen states. Extenders used for diluting bull semen typically are egg yolk and/or skimmed milk-citrate/Tris-based buffers with added simple sugars and antibiotics, with or without cryoprotectants depending on whether they are one-step or two-step extenders. These components provide an acceptable buffering capacity and osmolality and energy in the form of metabolizable substrates, minimize bacterial growth, and protect sperm from decreases in temperature.

As mentioned earlier (in the section "Mechanisms of Cell Injury During Cryopreservation"), sperm can suffer cold shock as a result of a sudden reduction in temperature, which causes structural and biochemical damage. This can be prevented by cooling semen slowly in the presence of protective agents. Phillips [25] first reported the value of adding egg yolk to bovine semen to afford such protection. Subsequent studies have shown that egg yolk not only increases the fertilizing ability of spermatozoa at ambient temperatures but also appears to prevent sperm cell damage during cooling and freezing [26–29]. Over the years the concentration of egg yolk added to extenders has been reduced from 1 : 1 (volume/volume) to 20–25% of volume as this has been shown to improve sperm survival [30, 31]. The low-density lipoproteins (LDLs) in yolk have been shown to play an important role in protecting sperm, with some studies reporting that LDL by itself is better than whole egg yolk in preserving sperm motility after freezing [32, 33]. Besides egg yolk, skimmed milk has also been found to be very efficient in protecting sperm during semen storage at 4 °C or in cryopreservation [34–36], and has the same composition as whole milk but contains less than 0.1% lipids (mostly triglycerides). The protective constituent of skimmed milk is not the lipid fraction but rather the protein constituents known as casein micelles. There are various hypotheses on how LDLs provide protection. It has been suggested that the phospholipid fraction of LDL protects sperm by forming a protective film on the sperm surface [37] or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process [38, 39]. Vishwanath et al. [40] suggested that egg yolk lipoproteins compete with detrimental seminal plasma cationic peptides (<5 kDa) in binding to the sperm membrane and thus protect the sperm. However, recent studies and research favors the idea that LDL interacts with the major proteins of bull seminal plasma and this interaction appears to be crucial for sperm protection [41]. A family of major proteins, known as *b*inder of *sp*erm (BSP), found in seminal plasma binds to sperm at ejaculation and modifies the sperm membrane by removing cholesterol and phospholipids. This may adversely affect the ability of sperm to be preserved. LDL from egg yolk and casein micelles and whey proteins in skimmed milk sequester these BSPs, thereby maintaining sperm motility and viability during storage [42].

Apart from egg yolk and skimmed milk, extenders used for diluting bovine semen contain various buffers, with phosphate being one of the earliest to be used. However, sodium citrate has largely replaced phosphate due to its superior ability to promote sperm survival at 5 °C. Citrate also improves the solubility of protein fractions in the egg yolk due to its chelating properties. Many zwitterionic buffers such as Tris [tris(hydroxymethyl)aminomethane], TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid], and Tris titrated with TES (TEST) have been developed and tested over a wide pH range and have proved comparable or superior to citrate buffers. Of these, Tris-based diluents combined with egg yolk have been tested extensively and are used universally for extending bovine semen. In addition, protein components of skimmed milk extenders have been thought to provide buffering capacity to semen diluents. Because of the potential risk of xenobiotic contamination, research has started focusing on animal product-free extenders such as coconut and soymilk with satisfactory results. A recent study comparing soymilk tris (SMT) extender with an egg yolk tris (EYT) extender showed no significant differences between sperm in EYT extender and SMT extender with regard to post-thaw motility, viability, membrane integrity, acrosome integrity, and cryocapacitation [43].

Spermatozoa require energy for motility and are capable of both aerobic and anaerobic metabolism [44]. Most diluents provide an energy source in the form of simple sugars such as lactose, mannose, fructose, and arabinose. Sugars

also add osmotic pressure to the medium and act as cryoprotectants. The main effect of sugars and polyols such as glycerol is their ability to replace the water molecule in the normally hydrated polar groups, which helps to stabilize the sperm plasma membrane during transition through the critical temperature zones [13]. Sugars, like glycerol, also increase the viscosity of the diluent and prevent the eutectic crystallization of solutes, increasing the glass-forming tendency of the medium, a property used increasingly in vitrification media [45].

Most commercial bovine semen extenders contain antibiotics to reduce the rate of bacterial overgrowth and its subsequent deleterious effect on semen quality. Bacteria are present in the genitalia and reproductive tract of bulls regardless of fertility status and can be difficult to screen, even with the most hygienic and sanitary collection procedures and processing. Moreover, the presence of egg yolk and other extending media provides a good nutrient environment for the growth of these organisms. Historically, penicillin and streptomycin have been the preferred antibiotics used in combination with bovine semen extenders as they are relatively harmless to sperm and, when combined, inhibit a broad spectrum of microorganisms. However, these antibiotics fail to control growth of organisms such as *Campylobacter fetus* subsp. *venerealis* and have questionable efficacy against *Mycoplasma* and *Ureaplasma* spp. which can survive the freezing process. Continued use of these antibiotics has led to development of bacterial resistance. Because of these drawbacks the current international standards for semen extenders favor protocols that include treatment of semen and extender with the antibiotics gentamicin, tylosin, lincomycin, and spectinomycin (GTLS), as they are more effective in controlling *Mycoplasma*, *Ureaplasma*, *Campylobacter fetus*, *Haemophilus somnus*, and *Pseudomonas* in bovine semen [46, 47]. Recent studies have shown that bacterial presence in semen can adversely affect the DNA integrity of sperm, and the rate at which this damage takes place correlates positively with the initial bacterial load and bacterial growth rate [48]. Longevity of DNA is also adversely affected in the presence of GTLS combination. Use of the quinolone class of antibiotics has been shown to increase sperm DNA longevity in semen samples containing bacteria and thus could be of interest in terms of promoting alternative cryopreservation strategies to increase reproductive outcome [49].

The use of antimicrobials is not without problems, as many may have a detrimental effect on sperm survival, and the choice of agents that can be added to semen extenders is limited in food-producing animals. More importantly, continued use of antibiotics can result in development of resistance within an animal species, thus leading to an urgent need to find alternatives to antibiotic usage within the AI industry. Several strategies such as strict hygiene during collection and processing of semen samples under laminar flow have been suggested. Cationic antimicrobial peptides that act through destabilization of bacterial cell membranes have been investigated as alternatives to antimicrobials in semen extenders with promising results [50]. Physical methods for removal of bacterial load such as colloid centrifugation of semen have been investigated for human boar and stallion semen [51–53]. This technique in combination with strict hygienic and aseptic handling has resulted in abolishing almost all bacterial load in semen samples and improving the post-thaw quality of frozen samples. Considering the sheer volume of bovine semen frozen, preserved, and transported worldwide, these methods properly implemented and employed could help by reducing the use of a staggering volume of antibiotics used in semen extenders, thus minimizing their entry into the food chain and preventing bacterial resistance.

Role of Cryoprotectants in Bovine Semen Freezing

In 1949, Polge et al. [54] made a pivotal discovery showing that the use of glycerol (a permeating solute) could provide protection to cells at low temperatures. This is often cited as the defining moment in the establishment of modern sperm cryobiology. The development of cryopreservation protocols for the bull to be used for AI in the dairy industry began in the 1950s. Bratton et al. [3] demonstrated in field trials that bovine sperm frozen to −79 °C and packed on dry ice could still yield high fertility. The discovery of the protective properties of egg yolk lipids and glycerol further aided the development of freezing extenders for cryopreservation of bull sperm. The sum of these discoveries led to the development of the Tris–egg yolk–glycerol method for freezing bull sperm, which has now become a standard [36, 55]. Cryoprotectants are included in cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing, and thawing of sperm cells [10, 56], and are classified as either penetrating or non-penetrating based on their ability to cross membranes. Penetrating cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol) cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased survival to cryopreservation [4]. They act like solvents and dissolve sugars and salts in the medium [56]. Non-penetrating cryoprotectants (egg yolk, non-fat skimmed milk, trehalose, amino acids, dextrans, sucrose) do not cross the plasma membrane and act extracellularly.

They may alter the plasma membrane or act as a solute, lowering the freezing temperature of the medium and decreasing extracellular ice formation [57, 58].

Glycerol, a penetrating cryoprotective agent, is the most favored of cryoprotectants, largely because of the ability of bovine semen to withstand much higher levels of glycerol compared with other species. Numerous studies have shown that glycerol yields better post-thaw motility, lesser membrane damage, and better survival rates compared with other cryoprotective agents [59, 60]. Glycerol readily enters the cell after its addition and acts to lower the freezing point of the medium to a temperature lower than that of water. This reduces the proportion of the medium that is frozen at any one time, reducing the effect of low temperature on solute concentrations and hence on osmotic pressure differences [1, 61, 62]. It also provides channels of unfrozen medium between ice crystals in which spermatozoa may exist while at low temperatures and acts as a salt buffering agent. Cryoprotective agents in general are believed to act by increasing the osmotic pressure of the extracellular fluid and hence draw water out of the spermatozoa, thereby decreasing the risk of formation of ice crystals and hence physical damage. However, they do not alleviate, and may even exacerbate, the problem of dehydration and increases in solute concentration. Glycerol and dimethyl sulfoxide can induce osmotic stress and toxic effects on spermatozoa, the extent of which vary according to the species and their concentration in the extender solution [56]. The concentration of glycerol in the diluent for optimal cryosurvival for bull semen ranges between 6 and 9%, with upper levels causing damage to sperm and lowering their post-thaw survival rate. While formulating semen extenders, glycerol may be added initially or later in a separate fraction after semen refrigeration. In the first situation, also known as the one-step method, the entire semen extender along with glycerol is joined after semen collection. In the second situation, or two-step method, a fraction of extender without glycerol is joined first. This is followed by refrigeration, after which the glycerolated fraction is joined just before semen freezing [63]. The protocols used for adding cryoprotectants and freezing are ultimately a compromise between the advantages and detrimental effects of their incorporation and may vary between individual breeding bulls in order to obtain optimal results.

Steps for Cryopreservation of Bovine Semen

Semen cryopreservation procedures have been evolving over the past few decades. However, despite continued advances in the development of newer extenders and cryoprotectants, the post-thaw motility of bovine sperm remains at around 50%. This is because semen cryopreservation involves several general steps, of which dilution, cryoprotection, cooling and freezing, storage, and thawing can affect sperm structure and function [64]. Bovine sperm are thought to be relatively more resistant to higher concentrations of cryoprotective agents and the freezing process. However, to optimize post-thaw motility and membrane stability rates, the semen collected should be of high quality and more detailed attention should be paid to evaluation techniques than with those performed during a routine breeding soundness examination. Similarly, individual differences can be observed based on sensitivity of semen to extenders and freezing techniques among bulls. Thus, allowances have to be made by packaging straws with more spermatozoa or by adjusting freezing protocols for individual bulls [65]. Certified Semen Services Inc. (CSS), a wholly owned subsidiary of the National Association of Animal Breeders (NAAB), was established to assure standards and authenticity of semen products. It is also responsible for disseminating information concerning the handling and processing of semen. There are currently two standard protocols approved commercially by the CSS for freezing bovine semen. The standard CSS protocol or the two-step method is the most common method utilized in the USA, whereas the one-step method (also known as the alternative CSS protocol) is more popular in Europe. We routinely employ the two-step method for bovine semen freezing, which is described in greater detail in the section 'Two-Step Protocol for Cryopreservation of Bovine Semen."

Dilution, Initial Cooling, and Packaging

After collection and evaluation, the semen should be extended to an appropriate dilution/concentration using various diluents/extenders. The dilution stage is performed to minimize any potential toxic effects of seminal plasma, to suspend the spermatozoa in the freezing diluent, and to extend the semen to allow maximum usage [66]. A wide range of diluents of varying composition have been used in successful freezing protocols but the basic requirements are the same in all cases. The diluent must maintain osmolality, pH, and ionic strength, provide an energy substrate, contain a cryoprotective agent, and usually contain antimicrobial agents. A number of commercially available diluents for bovine semen seem to adequately fulfill these requirements (Table 78.1). The most commonly used diluents contain Tris–egg yolk/milk–glycerol as buffers and cryoprotective agents, although there are some extenders

Table 78.1 Some common commercially available extenders for bovine semen cryopreservation and their components.

Extender name and manufacturer	Diluents used	Cryoprotective agents	Freezing protocol	Antibiotics
Bovine Semen Extender by Agtech®	Tris buffer + citric acid + fructose	Glycerol and egg yolk[a]	Two step	GTLS[b]
Biladyl® by Minitube	Tris buffer + citric acid + sugars	Glycerol and egg yolk	Two step	GTLS
Triladyl® by Minitube	Tris buffer + citric acid + fructose	Glycerol and egg yolk	One step	GTLS
Triladyl CSS by Minitube	Tris buffer + citric acid + sugars	Glycerol and egg yolk	One step	None[c]
AndroMed® by Minitube	Tris buffer + citric acid + fructose + antioxidants	Glycerol and soybean lecithin	Two step	GTLS
AndroMed CSS by Minitube	Tris buffer + citric acid + fructose + antioxidants	Glycerol and phospholipids (animal origin free)	Available as one step and two step	None
BioXcell® Bovine semen preservation medium by IMV Technologies	N/A	Glycerol and soybean lecithin	Available as one step and two step	GTLS
Biociphos-Plus® by IMV, L'Aigle, France	N/A	Glycerol and soybean lecithin	One step	GTLS

[a] Egg yolk and water to be added prior to the addition of semen.
[b] Gentamicin sulfate, tylosin tartrate, lincomycin, and spectinomycin combination.
[c] None: one-step extenders require additional quantities of GTLS antibiotics usually supplied by the manufacturer.

available now in the market that are free of products of animal origin and contain phospholipids instead. These have been manufactured given current needs for disease control and therefore the avoidance of biologically derived substances in cryoprotective media. The diluents are usually available as a mixture of buffers and antibiotics to which the egg yolk is added at the time of semen extension. Similarly, depending on the process (one-step or two-step method), the glycerol fraction of the extender is added initially or later after refrigeration and cooling of the extended semen. The more commonly used two-step protocol will yield a final concentration of 20% egg yolk, 7% glycerol, 50 µg tylosin, 250 µg gentamicin, 150 µg lincomycin, and 300 µg spectinomycin in each milliliter of extended frozen semen. The one-step protocol yields an antibiotic concentration double that of the two-step procedure.

Dilution rates for bovine semen may vary depending on the concentration of spermatozoa in the ejaculate. In general, high dilution rates (typically 1 : 5 semen/diluent) are usually sufficient as the insemination doses used to achieve fertilization in the bovine are surprisingly low (typically 10–15 million) compared with those required for other species such as the horse and pig. However, care has to be taken to expose semen to an appropriate volume and concentration of extender, as overdilution may in fact result in loss of motility and increase in vital staining [67]. This phenomenon is known as the "dilution effect" and represents loss of cell viability, probably through leaching of structural components of the cell membrane [68]. The quantitative difference in the ratio of semen to extender between species is an important determinant of the fertility of cryopreserved semen [4]. In species where a large number of spermatozoa are required for conception, the dilution rates are correspondingly low, which means spermatozoa are more tightly packed, resulting in lesser tolerance to the cryopreservation process and poor survival rates.

Semen thus diluted is then cooled slowly to 4 °C over a period of two hours. This helps in reducing the metabolic rate of sperm greatly and minimizes the damage due to cold shock, apparent when semen is cooled below 15 °C [66]. Metabolic rate is believed to double for every 10 °C rise in temperature; hence cooling semen from body temperature of about 39 °C to refrigerator temperature of 4 °C reduces the metabolic rate to about one-tenth that at body temperature. Though bull spermatozoa are sensitive to cooling to refrigerated temperatures, the phospholipid components present in the egg yolk and whey proteins in skimmed milk play an important role in reducing the damage and also permit faster cooling. Once the partially diluted semen attains a holding temperature of 4 °C, it is extended to its final desired concentration by addition of the glycerol fraction if the two-step protocol for cryopreservation is followed. (Note that in the one-step protocol, semen is extended to its desired concentration and includes addition of glycerol prior to the cooling process.) The step involving addition of glycerol is sometimes erroneously

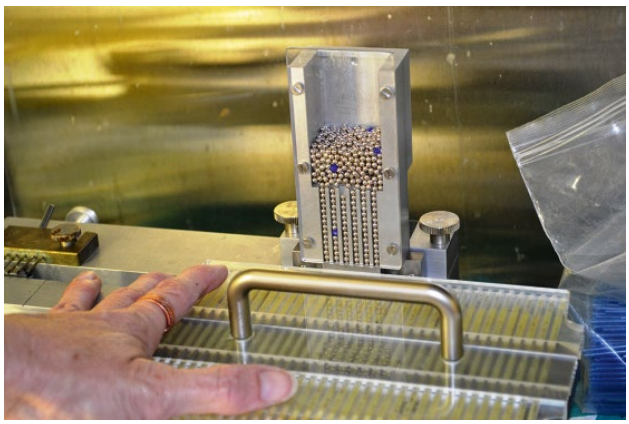


Figure 78.1 Sealing of straws using metal beads.

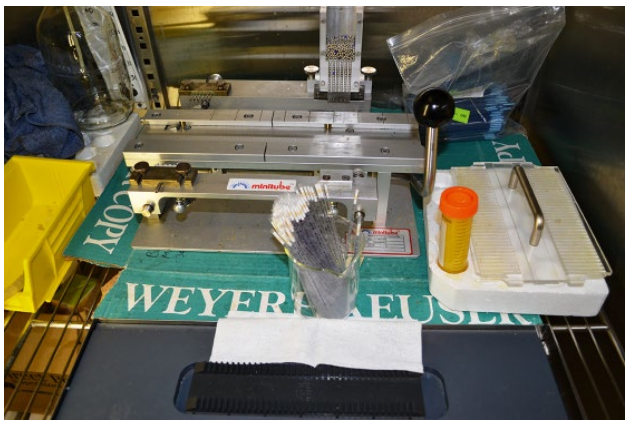


Figure 78.2 Cold cabinet used for semen cooling and packaging.

referred to as the glycerol equilibration period. Since glycerol has the ability to equilibrate quite rapidly across the cell membrane at 5 °C, it can realistically be added at any time during the cooling period [29, 69]. The equilibration period is thought to involve a process of membrane stabilization. Semen thus extended to its appropriate concentration contains around 7% glycerol and is further held at 4 °C for a few hours. During this holding period semen may be filled, packed, and sealed in 0.25- or 0.5-ml (standard size used in the USA) polyvinyl straws for further processing (Figure 78.1). All procedures involving the handling of cooled semen are performed in a cold-handling cabinet in order to maintain the semen equilibration temperature (Figure 78.2).

Cooling Rates and Freezing

During the process of freezing and thawing semen, spermatozoa are prone to damage caused by the rapid and dramatic changes in the physiochemical conditions occurring during cooling and ice formation. The extent of damage brought about by these abrupt changes is strongly affected by the cooling rate and composition of the cryoprotective medium. Cooling rates for cryopreservation of semen have been determined for various domestic species of animals and humans, sometimes in combination with the cryoprotectant concentration. Optimal rates ideally should fall in ranges that are neither too fast nor too slow. During the process of cooling and freezing, the first change that spermatozoa have to cope with is reduction from body temperatures to near freezing point of water [1, 6]. During semen refrigeration with slow cooling rates (0.5–1 °C/min), temperature reduction induces stresses on membranes, probably ascribed to the phase changes in lipid bilayers and altered functional state of membranes [6]. This is believed to impair the function of membrane proteins that are necessary for structural integrity (cytoskeleton) or ion metabolism (ion pumps) [6]. Major changes in bull spermatozoa during this phase occur in the vicinity of 15–5 °C but do not occur below 0 °C [6]. A second major change in the environment is coincident with the formation of ice crystals after the solution is cooled to a temperature between −5 and −15 °C. The release of the "latent heat of fusion" then causes the sample to warm up abruptly, until the freezing/melting temperature of the solution (of the remaining unfrozen fraction) is reached [6]. This results in spontaneous ice nucleation as the solution is supercooled and ice crystals then grow rapidly in all directions. Ice formation will depend on the rate at which the medium is further cooled and what remains is the so-called unfrozen fraction in which all solutes are confined. The osmolality of this fraction increases due to the increased concentration of glycerol, sugars, and salts and causes a rapid efflux of water from the cells or cellular dehydration. Fast cooling at such a stage can in fact lead to very sudden changes in size, shape, and ultrastructure of cells and lead to structural deformation. Spermatozoa may find themselves confined to very narrow channels of unfrozen solution, squeezed between growing masses of ice, which could lead to mechanical stress in cells. As cooling continues, these processes continue until the viscosity of the unfrozen fraction becomes too high for any further crystallization.

A two-factor theory accounts for the effects of cooling rates on spermatozoa [70, 71]. Slow cooling rates expose spermatozoa to "solution effects" such as increasing salt concentration, increasing osmolality, and changing pH, whereas fast rates may not allow intracellular water to pass out, leading to intracellular ice nucleation. Optimal cooling rates for semen before freezing in domestic species are generally considered to be in the range 10–100 °C/min [66], resulting in good survival rates after thawing. In bulls, cooling rates can range between 50 and 60 °C/min, although

Woelders et al. [13] have suggested cooling rates as high as 100 °C/min with optimal results. One advantage of such high cooling rates could be a decrease in glycerol concentration in the medium, since glycerol is thought to protect from effects of slow cooling damage. This would also help avoid the osmotic damage caused due to the high glycerol concentrations used presently for freezing bovine sperm. However, there are technical difficulties in achieving such uniform cooling rates for straws independent of their position in the freezing apparatuses [13].

Currently there are two options available for freezing bovine sperm: controlled freezing by use of automated programmable freezers, and conventional vapor freezing (non-programmable) by holding semen straws over liquid nitrogen vapors at a specific time and distance. Commercial semen freezing machines (Figure 78.3) offer the option of accurately controlling the temperature inside the cooling chamber as well as programming the time course of the temperature. However, because semen straws are placed in more than one layer, this contributes to considerable variation in cooling rates in individual layers within a freezing cycle and the time course of temperature inside the straws may be different due to the generation of latent heat of fusion. Also, programmable freezers are more expensive, and they do not necessarily yield more satisfactory results, especially for experienced technicians and cryobiologists. Conventional vapor freezing or non-programmable freezing can be performed by partially filling a non-ventilated Styrofoam box with liquid nitrogen and freezing straws by arranging them on a rack placed inside the box (Figure 78.4). The height of the straws above the liquid nitrogen determines the rate of heat exchange, with the rate of decrease in temperature being between 150 and 300 °C/min. In addition, the advantage of conventional freezers is that all the straws in any given freezing cycle are subjected to the same cooling rates, because the straws are placed in one layer above the liquid nitrogen level. In non-programmable freezers the cooling curve (the time course of cooling and freezing) is by default the form theoretically predicted to be optimal for slow freezing [72]. This results in relatively low cooling rates directly after ice formation begins and higher cooling rates later. The overall steepness of the freezing curve can be adjusted in non-programmable systems by choosing the height of the straws above the liquid nitrogen, which is proportional to the temperature of the vapor around the straws.

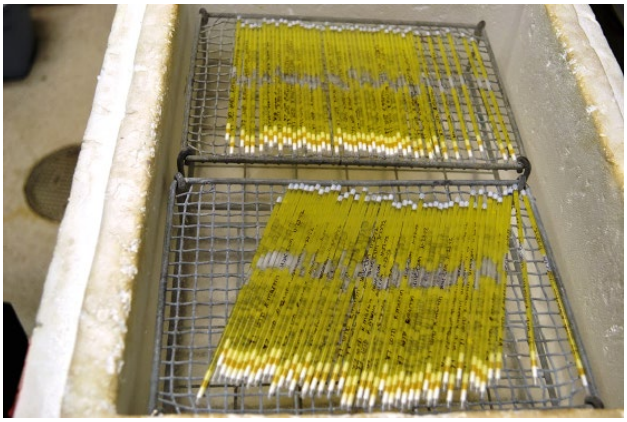


Figure 78.4 Vapor freezing by placing straws on a rack 5 cm above liquid nitrogen level in a Styrofoam box.



Figure 78.3 Programmable freezers for semen straws as well as embryos.

Semen Thawing and Storage

Frozen spermatozoa can experience damage during the thawing process as there is a complete reversal of the cellular events that occur during freezing. During slow thawing, the recrystallization of ultramicroscopic ice crystals to form comparatively large ice crystals [6, 13] can occur when spermatozoa pass through the critical temperature zone of −5 to −15 °C [73] and can be harmful to spermatozoa. Faster thawing rates (optimum: 37 °C for at least 45 seconds) limit the time for recrystallization and thus increase the survivability of sperm. However, when the duration of thawing is insufficient for the outflow of excess cryoprotectant from the cell, it suffers osmotic stress and the spermatozoa swell and lyse as the medium becomes abruptly diluted by the melting of extracellular ice [74]. The thawing rate can be influenced by factors such as the temperature and nature of the environment (air or water bath) and the thermal conductivity of the packaging as related to the diameter of the lumen of the packing. Faster thawing rates may be achieved by using higher temperatures for shorter periods of time (e.g. 65 °C for 5 seconds); however, care needs to be taken to avoid overheating the semen.

Optimal storage and handling of semen straws is a crucial factor that can contribute significantly toward the success of any AI program. Semen is usually packaged in 0.5-ml straws, although some organizations (especially in Europe) prefer packaging in 0.25-ml straws. Regardless of size, the handling procedure is common and straws are usually placed in goblets which in turn are clipped onto a metal cane. The canes are thus identified properly with the bull's name or identification number and are stored in canisters of specifically designed liquid nitrogen tanks. Though a detailed description of the construction of a liquid nitrogen tank is beyond the scope of this chapter, the need for proper care in storage and handling of these tanks cannot be overemphasized. Though semen tanks are designed to be sturdy, they are more fragile than they appear and require regular care and attention to prolong their productive life. Failure to do so can lead to a lost inventory or reduced pregnancy rates because semen quality is compromised. Tanks should be stored in a dry, well-lit, and well-ventilated area away from direct sunlight. They should be kept elevated above the concrete floor or other wet and poorly ventilated surfaces as corrosion of the outer shell shortens the functional life of the tank and can possibly cause tank failure. One of the key external signs of tank failure is frost build-up (especially around the neck), indicating loss of vacuum. The level of liquid nitrogen in the tanks needs to be regularly monitored and topped up when necessary. While retrieving straws from the tank, the canisters should be kept suspended as low as possible in the tank in order to maintain the temperature of the straws at a constant −196 °C. When straws cannot be retrieved from or located in canisters in less than 10 seconds, the canister should be lowered back into the tank and held for a period of at least 20 seconds to recool before trying again.

Stepwise Procedure for the Two-Step Protocol for Cryopreservation of Bovine Semen

Note that if directions for the extender supplied by the manufacturer differ, then it is advisable to follow those.

1) Prepare Fraction A (containing buffers and added 200 g of egg yolk) of the extender and place in the incubator before semen collection. Place Fraction B (containing glycerol) of the extender in the refrigerator.
2) After examining and assessing semen quality and concentration, dilute the semen slowly with a small volume of the warmed Fraction A extender in a 50-ml dilution tube. Depending on the calculated number of straws to be processed, the ejaculate can be extended with Fraction A to 50% of the calculated final volume.
3) The extended semen can be cooled to 4 °C by first keeping at ambient room temperature and later placing in a refrigerator or a cold room. The sample should be held at 4 °C for a minimum of two hours. The extended semen can also be placed in a water bath having the same temperature, and then placed to be cooled to 4 °C. This helps with the cooling rate.
4) Fraction B, which has been previously cooled to 4 °C, can now be added to the extended cooled semen in a 1 : 1 ratio gradually over a period of 20 minutes.
5) The semen now extended to its final concentration should be placed again in the cold room or cabinet to equilibrate for a period of four hours. During this time straws may be labeled with the appropriate information (e.g. breed, registration, number, name, date). They are filled, sealed, and placed on racks for freezing and counting. Straws should be shaken so that the air bubble is in the center after filling and before freezing. All procedures should be performed at 4 °C in a cold room or cooling cabinet.
6) Straws are frozen by placing them on a rack at a distance of 5 cm above the liquid nitrogen level in a Styrofoam airtight container. They are thus held over the liquid nitrogen vapor for about 15 minutes and then plunged in liquid nitrogen (Figure 78.5).
7) Straws are loaded into canes (labeled with the bull information) and transferred to liquid nitrogen canisters. One straw should be thawed for assessing post-thaw motility as well as concentration. Ideally the concentration per straw should be equal to one insemination dose (10–15 million sperm). This may be adjusted to bull fertility data if available.

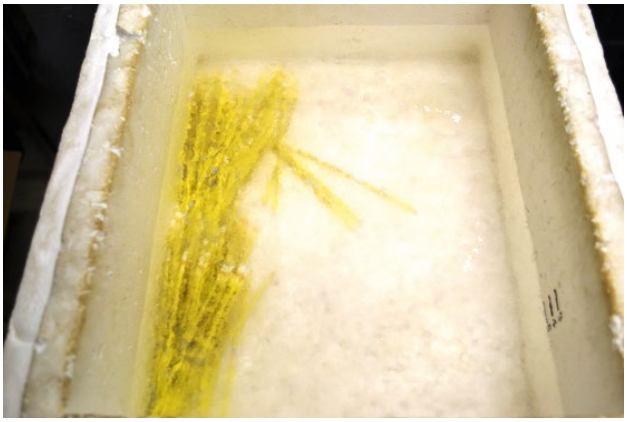


Figure 78.5 Straws plunged into liquid nitrogen.

Newer Advances in Bovine Semen Cryopreservation

Over the years several advances in the field of cryobiology have benefited the bovine AI industry greatly. These have resulted in the development of superior semen diluents,

standardized optimal freezing protocols, predictable fertilizing ability of sperm through different semen viability tests, and a better understanding of the pathophysiology of cryopreservation-related sperm damage.

Recent studies have shown that addition of 8% coconut oil as a source of lauric acid to egg yolk based-extender [75], 5 ng/ml α-linoleic acid to the BioXcell extender [76], and 20 ng/ml arachidonic acid to tris-citric acid extender [77] enhanced the post-thaw parameters of spermatozoa following cryopreservation. Iodixanol, a medium used for density gradient centrifugation, when added to semen extender has shown to enhance sperm motility post-thaw via reducing the formation of ice crystals during the freezing process [78].

The current need for disease control and the avoidance of biologically derived substances of animal origin in cryoprotective media have led to development of coconut milk and soy lecithin-based semen extenders, which have been shown to have comparable protective properties as egg yolk and skimmed milk. However, the quality of soybeans can vary from batch to batch and can affect the quality of the extended and subsequent fertility rates [79]. Substances such as synthetic liposomes [80] and cholesterol-loaded cyclodextrins [81] have been shown to afford better protection to sperm during cryopreservation and have yielded increased post-thaw survival rates. A recent research [82] evaluated the ability of Tris-glycerol extender pretreated with cholesterol-loaded cyclodextrins to preserve sperm motility post-thaw as well as the *in-vitro* fertilization potentials in absence of any source of animal proteins (milk or egg yolk). The cholesterol-loaded cyclodextrins not only were able to preserve sperm motility comparable to rates achieved using conventional egg-yolk based extender, but also had similar cleavage and blastocyst rates.

Antioxidants are molecules that inhibit the formation of ROS and lipid peroxidation. Superoxide dismutase, glutathione peroxidase, and catalase are the well-known antioxidants that are significant for sperm function because they protect sperm cells from oxidative stress [83]. However, endogenous antioxidants present in seminal plasma are not enough to protect spermatozoa against the stresses endured during cryopreservation. Hence, addition of substances demonstrating antioxidant properties to semen extenders to protect the sperm from ROS and ultimately lipid peroxidation-associated damage has been investigated. Glutathione (GSH) has been shown to increase motility, integrity of the plasma membrane, and cell viability of buffalo semen via its action against free oxygen radicals [84]. Similarly, resveratrol and vitamin E supplementation have shown to improve post-thaw motility and viability of spermatozoa through their roles in protecting sperm chromatin and membranes and maintaining membrane potentials in ram and boar semen [85, 86]. Curcumin and erythritol and *n*-3 fatty acids combined with α-tocopherol [87] have shown promising results pertaining to better cryosurvival of spermatozoa by altering membrane lipid composition. Supplementation with antioxidants such as methionine, carnitine, and inositol prior to the cryopreservation process has been shown to protect DNA integrity against cryodamage [88].

Several advances have been made or are under investigation currently with the aim of solving or mitigating old issues associated with conventional semen cryopreservation techniques. Vitrification is a rapid freezing method requiring only a brief manipulation procedure, thus decreasing the probability of chilling injury (cold shock) [89]. Semen vitrification has been carried out successfully in various species including human, equine, caprine, and canine. Human studies have found that semen functionality was better preserved via vitrification as compared to the standard slow freezing techniques, allowing a higher percentage of motile sperm to retain intact mitochondrial function and DNA integrity [90]. Its use in the bovine industry is non-existent and still in the experimental phase. This technique, however, has the potential of being a viable alternative in the near future and seems to have biological, operational, and economic advantages over the conventional freezing methods. Lyophilization or freeze-drying techniques for equine spermatozoa and subsequent birth of live foals [91] have opened a whole new perspective for cryopreservation of sperm. Though no live calves have been reported yet, lyophilization of bovine sperm may offer an attractive alternative to liquid nitrogen storage of sperm. Since lyophilized sperm can fertilize an oocyte only via procedures such as intracytoplasmic sperm injection, and requires fewer numbers per dose, the number of doses obtained per ejaculate could literally be in the hundreds of thousands. Lyophilization of semen may help overcome several drawbacks associated with conventional cryopreservation such as high maintenance costs and problems associated with transportation of the frozen semen, as well as accidental loss due to improper temperature control. Microencapsulation is yet another alternative technology that has recently been perfected for bovine semen, which maintains sperm viability by prolonging sperm survival at low temperatures [92, 93]. This technology consists of immobilization and cryopreservation of semen within a solid gel made of calcium alginate, thus increasing the availability of fertile spermatozoa in the reproductive tract by release of gametes over time. Availability of discrete populations of viable sperm over a prolonged period of time for fertilization would help in making the choice of time to perform AI less critical [92].

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79

Utilization of Sex-Selected Semen

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Introduction

Gender selection of offspring has considerable benefit for livestock management systems. Planning the sex of the offspring prior to conception is the most cost-effective means of achieving the desired outcome. Utilization of gender-selected semen allows production of male and female offspring to take advantage of sex-limited and sex-influenced traits [1]. To be widely used, gender preselection should be effective and efficient, result in acceptable fertility, and be reasonably inexpensive and convenient [1]. In mammals, sex is determined by gonosomes: an XX chromosome combination determines a female, whereas an XY results in a male. Since X and Y chromosomes differ significantly in length, discrimination of X and Y chromosome-bearing sperm populations is possible due to the difference in their DNA content. Several methods to separate X and Y chromosome-bearing sperm populations of ejaculates have been investigated. The only accurate and potentially cost-effective approach for achieving gender selection currently involves separating X from Y chromosome-bearing sperm by flow cytometry, followed by its use for artificial insemination (AI) for breeding or multiple embryo production systems, or for *in vitro* fertilization (IVF) with subsequent embryo transfer programs [2, 3]. This method can produce populations of X or Y sperm with up to 90% accuracy and subsequent offspring whose phenotypic sex is consistent with the initial accuracy of the sex-sorted sperm population. Limitations of the technology are associated with the number of sperm required for fertilization, timing of insemination, individual bull variation in sexed-sperm sorting, and subsequent production of offspring. This technology examines each sperm separately for its DNA content, thus limiting the number of sorted X or Y sperm to approximately 5–6 million sperm per hour [2].

Sorting Sperm by DNA Content for Gender Selection

Flow cytometric examination of the DNA content of X and Y chromosome-bearing sperm of cattle (*Bos taurus* and *Bos indicus*) indicated a difference in their DNA content. The difference in total DNA content between those from bulls and those from cows is approximately 4.2% [4]. This average DNA content difference is sufficient to detect by flow cytometric systems [5–7] (Table 79.1) and subsequent sorting of X and Y chromosome-bearing sperm.

The sperm sex-sorting procedure involves several steps. Briefly, sperm DNA is quantitatively stained with Hoechst 33342 and the sperm are then forced in a stream in front of a laser beam at specific wavelengths [3, 8, 9]. The illuminated stained sperm emit a very bright-blue fluorescence. This fluorescence is rapidly measured using a photomultiplier tube as the sperm flow in single file in front of the tube. A computer is used to rapidly analyze the relative fluorescence of the X and Y sperm populations as they flow through the instrument in a fluidic stream. A crystal vibrator is used to break the stream into individual droplets, many of which contain a sperm. The fluorescently stained sperm are sorted by DNA content by placing opposite charges on droplets containing X sperm from those containing Y sperm (Figures 79.1 and 79.2). The droplets fall past positive and negative electrical fields, and since opposite charges attract, the droplets separate into two streams for collection. A third stream of uncharged droplets is discarded, as these droplets have sperm that could not accurately be sexed (over half), no sperm, rarely two sperm, and dead sperm (gating out dead sperm is a valuable additional benefit). This sperm sorting technique is known as Beltsville Sperm Sexing Technology [3, 8, 9].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

Table 79.1 Average DNA content difference (%) in X and Y chromosome-bearing sperm of different breeds.

Holstein	4.98
Jersey	4.24
Angus	4.05
Hereford	4.05
Brahman	3.73

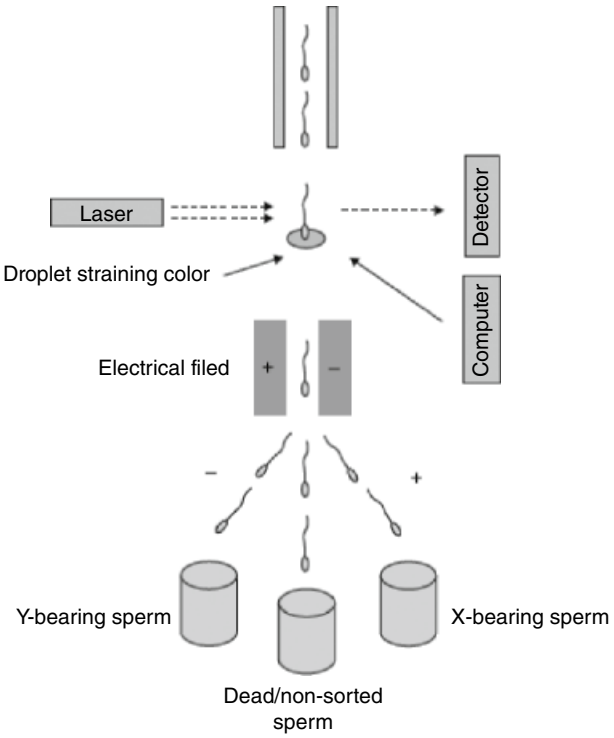


Figure 79.1 Schematic of flow cytometry for sorting X- and Y-bearing bovine sperm.

Commercialization of Bovine Gender-Selected Semen

Advances in sex-sorting sperm have enabled its incorporation into commercial reproductive management. Currently, gender-selected semen is more applicable on a large-scale basis to the dairy industry than to the beef industry. Sexed semen will contribute to increase profitability of dairy and beef cattle production in several ways. It could be used to produce offspring of the desired sex from a particular mating to take advantage of differences in value of males and females for specific marketing purposes. Dairy herds are more interested in producing replacement heifers, as dairy bull calves are inherently less valuable. Advantages are production of more replacement heifers, thereby enabling greater selection pressure, contributing to more rapid



Figure 79.2 Genesis III sperm sorter for gender selection. *Source:* Image courtesy of XY Inc.

genetic gains [10]. There are opportunities for herd size increase, sale of more females, and simultaneous increase in milk production and decrease in milk price (the latter is an advantage for consumers). Furthermore, once replacement heifer needs are met, dairy producers could use sexed semen to produce beef crossbred bulls from the remainder of their cow population. Seed-stock dairy producers gain their income from selling genetically superior animals in addition to selling milk. Their goal is to maximize genetic merit of breeding stock for economically important commercial production traits in a cost-effective manner. Sexed semen will be useful to seed-stock breeders if it allows them to increase rate of response to selection or if it reduces the cost of achieving genetic change.

In order to produce genetically superior replacement heifers using sexed semen, commercial dairy farmers should first rank their cows according to estimated genetic and economic merit for dairy production traits and then use X chromosome enriched semen from genetically superior bulls to inseminate a sufficient number of the highest-ranking cows to produce the needed number of replacements. By selecting only the best cows to produce replacement heifers, farmers would increase selection pressure for dairy production traits. Methods to assess the

economic impact, generated through increased response to selection among dams of commercial cows, of a unit of semen with an increased proportion of X chromosome-bearing sperm have been discussed [11]. It was reported that the predicted impact on annual genetic progress for sexed semen technology is a 9% [12] or 15% [13] increase compared with a mating system using artificial insemination (AI) with conventional semen.

The commercial beef cattle farmer produces calves for eventual sale as slaughter animals and for female herd replacements. Depending on the planned role for a calf, males and females are of quite different value, so sexed semen potentially is a powerful technology to affect genetic and economic efficiency. With beef cattle herds, the value of shifting the calf crop gender ratio to favor bulls or heifers depends on herd genetics and marketing plans. For beef cattle seed-stock producers, bull sales to commercial cattle producers are often the most significant source of revenue. Replacement heifers are also important for genetic improvement. For beef cattle producers who depend on bull sales and require fewer replacement heifers, utilization of male (Y-bearing) gender-selected semen could be practical. Steers are usually heavier at weaning and more valuable than heifers. Thus, for commercial cattle producers who market feeder calves, utilization of Y-bearing semen in a commercial setting would lead to more steer calves that could offset increased semen cost. Furthermore, if heifers are retained as replacements, individual hand-selected matings could be done to produce heifers from more maternal dams and bull calves from terminal crosses. It should also be noted that in some beef cattle breeding programs, heifers are more valuable than their male counterparts.

Artificial Insemination

Sexed semen technology became practical with the live birth of offspring of the predicted sex [14]. Continued research recognized the ability of flow sorting to consistently produce approximately 90% gender-selected offspring and facilitated eventual commercial adaptation [15, 16]. The technology was introduced into commercial application in the UK in 2000. Currently, several US AI centers have this technology and market sexed semen from their bulls worldwide.

Conception rates to AI in cattle are related to sire, semen processing, sperm dosage, timing of insemination, and service number. Despite successes, the invasive nature of the sex-sorting procedure has detrimental effects on sperm viability and quality [17, 18]. In addition, for economic reasons, the number of sperm in sex-sorted samples is reduced to approximately 2 million sperm per dose [18]. In breeding programs that use conventional semen, some factors that cause differences in fertility are amenable to increased sperm numbers in an insemination dose (compensable), whereas others are not (uncompensable) [19]. An extensive insemination dosage trial concluded that the compensable component is mostly satisfied at total sperm dosages of approximately 4.0 million; however, considering differences among sires, the range is 1–11 million sperm per dose. Seidel [18] and Schenk et al. [16] predicted that commercial application of doses of 2.1 million sex-sorted sperm in Holstein heifers would yield conception rates approximately 75–80% of those obtained with conventional semen, consistent with field results in the USA [20]. Increasing sperm number in the insemination dose seems to be a plausible solution to increase fertility. A study compared conception rates for 2.1 or 10 million sperm dosages of sex-sorted or conventional sperm, respectively [21]. Across herds and sires, the conception rates of sperm dosage by semen type combinations were 38 and 44% for 2.1 and 10 million sex-sorted semen, and 55 and 60% for 2.1 and 10 million conventional semen dosages, respectively. A marginal improvement in conception rates was observed with an insemination dose of 10 million for some sires, suggesting that a portion of the decrease in conception rates with sex-sorted sperm may be compensable. However, the conception rates achieved with 10 million gender-selected sperm are not comparable to either dosages of conventional semen in this study. Therefore it implies that a portion of the decrease in the conception rate using sex-sorted semen is induced by the staining and/or sorting procedure. Further, although the sorting method is standardized, bull-related dilution effects may contribute to the decreased fertility independent of sorting stress [22], but this remains unproven as insemination of high numbers of sex-sorted sperm (10 million) led to reduced pregnancy rates compared with unsorted sperm inseminated at the reduced dose (2.1 million) [21]. Further, reducing the available insemination sex-sorted semen dose to 20% to achieve a 6% increase in pregnancy rate is a poor use of genetic potential [21].

Current recommendations for use of sex-sorted semen in a commercial operation are as follows:

1. Use only in herds with AI pregnancy rates of 60% or more with conventional semen.
2. Select healthy cycling females with good body condition, preferably heifers.
3. Inseminate only animals observed in standing estrus. If using fixed-time AI, make sure a high percentage of the animals were in estrus beforehand.
4. Inseminate heifers with sex-sorted semen once and subsequently with conventional semen if they do not conceive.

5. Be extremely careful with semen thawing and handling.
6. The inseminator should have experience and be known to achieve good pregnancy rates.

At present, the recommendation for use of sex-sorted semen is restricted to females exhibiting estrus [23]. However, the use of sex-sorted semen in conjunction with estrus synchronization for a timed AI has huge commercial appeal. Besides, estrus synchronization schemes are of particular value for insemination with sex-sorted sperm, as highly accurate insemination time is extremely important. The current focus is to develop an optimal protocol for fixed-timed AI of sex-sorted sperm. Insemination with sex-sorted sperm closer to ovulation [16, 24, 25] and insemination of females with large follicles at the time of AI [23] increased the likelihood of pregnancy. Additionally, uterine horn insemination could improve the conception rate when using sex-sorted sperm [15, 26, 27]. However, placement of sex-sorted sperm deeper into the uterus did not increase pregnancy rates to timed AI [23].

Multiple Ovulation and Embryo Transfer

Several studies have reported *in vivo* embryo production from superovulated cows and heifers following insemination with sex-sorted sperm. In many of those studies, there was a reduced fertilization rate and increased percentage of degenerate embryos from sexed sperm, which resulted in fewer recovered transferable embryos [28–30]. Suboptimal sperm quality and improper timing of AI were regarded as major factors contributing to fewer embryos per flush from sorted sperm [28, 29]. In contrast to single-ovulating cattle, multiple inseminations are believed necessary to cover the interval required for multiple ovulations [31]. Because the number of sperm per insemination dose is limited by the high cost of sexed semen, multiple inseminations with low dose (2 million) sorted sperm have been adopted [29, 30]. When donors were inseminated with sexed semen 12 and 24 hours after detected estrus, the number of transferable embryos was increased compared with donors receiving only one dose of sexed semen [29], indicating that two inseminations of sexed semen were more effective than one insemination at attaining embryo production characteristics similar to those for conventional semen. However, another study claimed that superstimulated donor cows inseminated four times had fewer Grade 1 embryos and more unfertilized ova with sexed versus conventional semen [32]. Additionally, when sexed semen was inseminated at various times after a detected estrus [30], the number of transferable embryos was decreased compared with donors receiving conventional semen.

Higher numbers of sperm are normally required for superovulated cows. The successful use of sexed sperm in bovine multiple ovulation and embryo transfer (MOET) schemes critically depends on the AI dose. The dose of sex-sorted semen that is used for regular AI, 2 million sperm, is insufficient [29]. The success with higher doses (10–20 million sperm) seems to depend on the sire or MOET conditions. Further, increasing the dosage to 20 million sex-sorted sperm lowered fertilization rates compared with controls [29]. Superstimulated heifers inseminated with 5 million sexed (X-sorted; $n = 5$) or unsexed ($n = 5$) frozen–thawed sperm from one bull at 12 and 24 hours after detection of estrus showed no difference in rates of transferable embryos (53.4 vs 68.1%) [33]. Furthermore, rates of degenerate embryos and unfertilized ova between sexed and unsexed sperm were 24.8 versus 26.6% and 21.8 versus 5.3%, respectively [33]. Producing higher numbers of good-quality transferable embryos and better pregnancy rates after transfer has great potential for a significant impact on the efficiency of utilization of sex-sorted sperm.

Contract matings between elite sires and dams that use MOET greatly increase the probability of producing a desirable offspring, even without sexed semen. Suppose that a MOET procedure resulted in three embryos that produced viable offspring. With conventional semen, the probability that at least one of the calves is of the desired gender would be 0.879 (1 minus the probability that all the offspring are the other gender). With 90% purity, the probability of producing at least one calf of the desired sex would be 0.999. For four viable offspring, the probabilities would be 0.934 and 0.999, respectively. Thus, it should be noted that whereas sexed semen increases the likelihood of producing at least one offspring with desired gender from a planned mating, the improvement is not huge.

The goal of most embryo transfer programs is to promote genetic gain from both the dam and sire. Utilization of sexed semen should yield such genetic gain equal to or better than conventional semen. A study estimated that the genetic trend in the entire population was improved by 3.0% when X chromosome-bearing semen was used on all production heifers without use of MOET in the nucleus herd and by 2.2% when MOET was performed on all bulls and dams [34]. When sexed semen was combined with MOET in the nucleus, the effect of using sexed semen was minimal, except when marker-assisted selection was applied [35]. Despite small genetic gain, a recent study reported a beneficial effect on the rate of inbreeding by using sexed semen in the breeding scheme [36].

In Vitro Fertilization

In vitro fertlization (IVF) requires low numbers of sperm for embryo production. Thus, use of sex-sorted sperm in IVF seems advantageous. Cleavage rates were similar to unsorted sperm, but blastocyst development with sorted sperm was approximately 70% of controls produced with unsorted sperm [37]. Further, there was a 0.5- to 1-day delay of embryonic development when oocytes were inseminated with sex-sorted sperm compared with unsorted sperm [37]. Similarly, in other studies, cleavage rates after IVF with sex-sorted sperm were 30% below those using unsorted sperm from the same ejaculate; blastocyst formation on day 8 was 30–40% lower than for the controls and the number of cell cycles was reduced [38, 39]. It was postulated that the impact of sorting on molecular aspects of sperm function, including capacitation, acrosome reaction, zona binding, and embryonic development, is a reason for decreased blastocyst production. Regardless, subsequent reports indicated negative effects of sorting on sperm integrity, but neither cleavage nor blastocyst rates were affected when sperm were centrifuged with a Percoll gradient [40]. IVF results are also affected by sperm concentration, which needs to be higher than for IVF with unsorted sperm, and by the hydrodynamic pressure used for sorting [41]. Additionally, there was a positive correlation between the percentage of progressively moving sorted sperm and blastocyst development [42].

Regarding differences in the expression of developmentally important genes, bovine IVF embryos derived from sex-sorted sperm had a reduction in the relative abundances of glucose transporter 3 (Glut3) and glucose 6-phosphate dehydrogenase (G6PD) compared with embryos derived from unsorted sperm, which may be deleterious to the developmental competence of embryos [43]. Further, it is interesting to note that there are significant sex-related (X- vs Y-bearing sperm) differences in day-7 blastocysts for several genes, namely glutathione *S*-transferase M3 (*GSTM3*), DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*), and progesterone receptor membrane component 1 (*PGRMC1*), plausibly contributing to delayed onset of the first cleavage [38, 44]. Additionally, blastocysts produced from sex-sorted sperm are observed with immature mitochondria and rough endoplasmic reticulum, as well as a lower percentage of intact nuclear envelope membranes than the non-sorted controls [42]. Although the sorting process has significant effects on sperm viability, and bull effects are obvious, compromised DNA does not affect reduced fertility, as most non-viable sperm, and those with fragmented DNA, are outgated during sorting. Regardless, further research is needed to investigate this phenomenon, as there is time-related DNA damage to sex-sorted sperm compared with non-sorted counterparts.

Reverse Sorting

Reverse sorting is sex-sorting of sperm from previously frozen samples and refreezing those samples after sorting. This technique allows previously frozen sperm to be separated for sex. This technique is useful for males that are located at a greater distance from a sex-sorting site or which are deceased and thus unable to provide fresh ejaculates for sex-sorting. The “reverse sorting” technique surmounts staining problems [45] by washing frozen sperm through a density gradient, thereby eliminating dead sperm, glycerol, and egg yolk, which may interfere with staining or negatively impact the remaining viable sperm [46, 47].

The first presexed offspring produced from reverse-sorted sperm were achieved in sheep following IVF and embryo transfer [48], and a few years later using AI [49]. Reverse-sorted bull sperm have poorer motility and viability *in vitro* compared with frozen–thawed controls [50]. Although presexed calves have been born following AI of reverse-sorted bull sperm, fertility is very low, with increased pregnancy loss [51, 52]. Greater success has been achieved with the use of reverse-sorted bull sperm in IVF, where cleavage and blastocyst production has been comparable to those obtained with sex-sorted frozen sperm and non-sorted frozen controls [53].

It was apparent that there was a difference among individual sheep with regard to success following reverse sorting. Although the reason for this difference between the species is the source of some disagreement [54], a better understanding would likely improve fertility of sorted and reverse-sorted bull sperm.

Effect of Sex-Sorting Procedure on Structural and Functional Parameters of Sperm

During the sorting procedure, sperm are subjected to several steps, including (i) high working pressures during hydrodynamic focusing and passage through the injection nozzle; (ii) DNA staining with the fluorescent dye bis-benzimide (Hoechst 33342); (iii) sperm exposure to wavelengths of the ultraviolet light spectrum; (iv) repeated exposure of sperm DNA to electrical currents and fields; and (v) subsequent passage through the electrostatic deflection field that may cause stress to sex-sorted sperm [55, 56]. Further impact on sperm quality may be related to preservation of stained sperm at low temperature until insemination. In addition, impact on the motility, viability, and functional integrity of sorted sperm may be attributable to a dilution effect following removal of seminal plasma. Perhaps a

major reason for a decrease in sorted sperm survival is production of reactive oxygen species (ROS) during several steps of the sorting process. Addition of sodium pyruvate and catalase to the dilution medium and freezing extenders exerted a positive effect on the motility, morphology, acrosomal status, and viability of the sex-sorted frozen–thawed sperm, confirming production of ROS in several steps during the sorting process [57].

Hoechst 33342 (bis-benzimide) is suitable for sperm DNA labeling as it preferentially binds to the minor groove of the DNA helix (for review see [58]). However, this dye is known to be mutagenic to cells and may induce disturbances in embryo development. Moreover, the fate of this dye transported by the spermatozoon into the oocyte and thereby into the embryo and offspring is an area that requires further investigation [59].

Assessment of sperm DNA fragmentation using non-sorted and sex-sorted sperm samples during a 72-hour incubation at 37 °C detected reduced DNA longevity in sex-sorted frozen–thawed sperm, with sperm DNA damage appearing between 24 and 48 hours [60]. Although the baseline DNA fragmentation level was higher in conventional frozen–thawed than in sex-sorted frozen–thawed sperm samples initially [60, 61], after approximately 30 hours of incubation there was cross-over in the tendency of DNA fragmentation for both conventional and sex-sorted sperm samples, with higher DNA fragmentation for sex-sorted than conventional semen [60]. Furthermore, it was concluded that sperm chromatin was more resistant to external stressors affecting sperm after sorting [60].

Cost and Revenues

Comparisons of heifers that were inseminated at their first AI breeding with either sexed or conventional semen (in both groups, all services after first AI used conventional semen) were made to evaluate subsequent reproductive performance and health, production, and reproductive performance during their first lactations (refer to [62] for detailed economic outcomes). Briefly, age of heifers at first insemination was 13.1 ± 0.1 and 13.8 ± 0.1 months for sex-sorted and conventional semen, respectively. Rearing cost from first AI to calving was greater for sex-sorted heifers than for conventional heifers (USD $775.3 ± 6.7 vs $750.0 ± 5.9). However, calf revenue tended to be greater for sex-sorted heifers ($142.0 ± 7.2 vs $126.7 ± 6.4) and cost per female calf produced was less for sex-sorted heifers than for conventional heifers ($–809.4 ± 10.8 vs $–1249.7 ± 10.9). Treatment did not affect calving difficulty, proportion of heifers needing assistance, and incidence of retained fetal membranes or metritis. Among heifers that conceived to first AI, those inseminated with sex-sorted semen were more likely to be culled within 30 days in milk (3.3 vs 1.6%) and tended to be more likely to be culled within 60 days in milk (5.5 vs 3.4%) compared with heifers inseminated with conventional semen, but overall replacement cost was not different ($136.8 ± 13.4). Total milk yield (9245.5 ± 84.7 kg) and income over feed cost ($554.7 ± 5.1) were not different. Overall economic return was greater for heifers inseminated with sex-sorted semen than conventional semen ($−83.7 ± 36.7 vs $−175.3 ± 33.4). Use of sex-sorted semen for first insemination of virgin heifers reduced the cost per female calf produced and increased the economic return during the first lactation. Female to male sex ratios were 0.86 : 0.14 and 0.48 : 0.52 for sex-sorted and conventional semen, respectively. Heifers inseminated with sex-sorted semen were more likely to deliver a dead calf (8.8 vs 3.4%), and thus the difference in proportion of heifers delivering a live female calf was lower than reported (sex-sorted 79.1%; conventional 47.2%).

Using sex-sorted semen, dairy herds could eliminate 70% of cows in third and greater parity as dams of replacements while maintaining heifer birth rates equal to those of conventional semen [63]. Elimination of older cows as dams of replacements reduces age of dam of replacements and increases genetic merit of dams by about $23. This change doubles the genetic change per year in the dam–daughter path compared with conventional semen, primarily due to shorter generation intervals [63]. Calculated economic values of sexed semen programs for dairy and beef heifers are available [64, 65].

Incidence of Stillbirth

Incidence of stillbirth was similar between heifers inseminated with conventional or sex-sorted semen [20, 66]. Furthermore, there were no breed differences for incidence of stillbirth among Holstein, Jersey, and Danish Red heifers inseminated with sex-sorted or conventional semen from the same bulls [67]. In contrast, a higher incidence of stillbirth following insemination with sex-sorted semen has also been reported [62]. In three years of using sex-sorted semen, total incidence of stillbirths was not influenced by the use of sex-sorted semen compared with conventional semen (10.4 vs 11.8%) [20]. Stillbirth for female calves resulting from sex-sorted semen (9.2%) is similar or slightly lower than conventional semen stillbirths. However, the incidence of stillborn births for male calves from sex-sorted semen (19.9%) is much higher than for conventional semen (12.9%). Even though heifers

delivering bull calves seemed to have a higher stillbirth rate, only 10% of calves born were male. However, this might be a concern where a higher sex ratio toward males is preferred. It was suggested that increased incidence of stillbirths may occur when pregnancies result from Y chromosome-bearing sperm that are mistakenly identified as X chromosome-bearing sperm during the sorting process due to the presence of aneuploidy, which would result in greater DNA content and increased risk for malformation [20]. In this case, it should be noted that the Y-bearing sperm sorted for male selection based on sperm having lesser DNA and the Y-bearing sperm that have aneuploidism with greater DNA will not be mixed with this Y-bearing sperm population as they will be selected out. Hence the stillbirth incidence will be similar to conventional semen when selection for male sperm is occurring.

SexedULTRA Technology

The fertility of sex-sorted semen in cattle has always been lower than that of conventional semen. Sperm compensable elements, higher sperm numbers, or a higher proportion of sperm with better morphological features, which normally would increase the fertility of a subfertile sire with conventional semen, did not yield better results with sexed sperm [17]. The reasons for the lower fertility of sex-sorted semen have been ascribed to the diverse biochemical changes that sperm undergo during the sex-sorting process. There are more than 20 different subprocesses involved in the sex-sorting procedure, including an extended holding time before staining, exposure to a laser beam to induce fluorescence, separation into X and Y sperm, and finally exposure to an electrical field for drafting as a relatively pure population into an appropriate vessel, all of which may contribute to the reduction in fertility [68].

SexedULTRA™, a new era in sexed semen technology, delivers conception rates up to 95% of those achieved through use of conventional semen [69]. The SexedULTRA™ process was devised as a system to simplify and optimize the media used for the sex-sorting protocol. The main changes included a modified protocol to pretreat sperm before the staining stage. In addition, the modified protocol includes a new staining medium with a balanced pH which keeps it stable across an extended period. To account for the low-dose freezing required for sex-sorted semen, the sheath fluid and the freezing medium were also modified. All these changes showed that SexedULTRA media had improved sperm motility as well as acrosome integrity compared with the XY technology at the same sperm concentrations [70]. In addition, SexedULTRA semen used in IVF trials resulted in a greater number of freezable embryos compared with the XY method (13.2 and 9.2%, respectively) [70]. Improvements made to the technologies and processes used to produce sex-sorted semen now allow units of SexedULTRA sex-sorted semen to be generated economically at 4.0×10^6 sperm per unit, as compared with the 2.0×10^6 cells per unit that had previously become the industry standard dose for sex-sorted semen [15, 17, 70, 71]. Extensive evaluations of SexedULTRA semen have indicated both improved fertility associated with the SexedULTRA process in comparison to sex-sorted semen produced using previous-generation technology [69] and improved fertility when using the higher dose rate of 4.0×10^6 cells per unit of SexedULTRA 4M™ [70]. Research efforts also suggest improved comparability between pregnancy rates obtained using SexedULTRA 4M sex-sorted semen and those obtained using conventional semen [70]. A large field trial was carried out in which semen from eight Holstein bulls was sorted by either the SexedULTRA or XY processing methods and used to inseminate 6930 Holstein heifers across 41 commercial herds in the USA. The SexedULTRA method resulted in a 4.5 percentage points (10.8% relative) improvement ($P<0.001$) in conception rate compared with the XY method (46.1 vs 41.6%, respectively) [70, 72].

Pregnancy rates to timed AI among mature beef cows following the 7-day CO-Synch + CIDR (controlled internal drug release) were reduced when using SexedULTRA 4M sex-sorted semen compared with conventional semen [73]. Further, pregnancy rates to AI were affected ($P = 0.04$) by the interaction of bull and semen type. Greater pregnancy rates were obtained with conventional semen versus SexedULTRA 4M sex-sorted semen when using semen from Bull A (64% [176/277] vs 36% [100/278]; $P<0.0001$) and Bull B (72% [200/277] vs 57% [156/276]; $P<0.01$), whereas pregnancy rates to AI did not differ between conventional and SexedULTRA 4M sex-sorted semen when using semen from Bull C (58% [149/258] vs 52% [131/254]). Pregnancy rates did not differ significantly between cows inseminated using a split-time artificial insemination (STAI) versus fixed-time artificial insemination (FTAI) approach, regardless of whether insemination was performed with conventional semen (65% [265/409] vs 65% [260/403]) or SexedULTRA 4M sex-sorted semen (50% [200/403] vs 48% [187/405]). Given the differences observed among bulls in this experiment, basic research efforts are warranted to better characterize sire fertility and develop predictive assessments of sperm fertility following sex sorting. However, as in a previous evaluation of SexedULTRA 4M sex-sorted semen, pregnancy rates to AI across

locations tended to be higher ($P < 0.1$) for heifers inseminated with conventional semen (60%; 257/429) compared with sex-sorted semen (52%; 218/422) [74]. Thus, pregnancy rates to timed AI with SexedULTRA 4M sex-sorted semen are near 50% and, as such, may be commercially acceptable in some beef production settings.

Conclusion

It is now clear that sexed semen is a commercial reality for cattle, deer, and sheep, and is nearing commercial application in several other species, particularly pigs, horses, and dogs. Reports on sexed semen being applied for conservation purposes have also become common in zoo animals and other wildlife, some of which are of threatened or endangered status [75].

The one aspect that has remained constant until recently and perhaps most concerning is fertility. In cattle and possibly other species it is believed that fertility of sexed semen is compromised by about 10 percentage points by an unknown mechanism that appears uncompensable by increasing the number of sperm per inseminate [17]. Sexing Technologies (Navasota, USA) has invested significantly in research and development in modifying processing media as well as biochemically altering the overall sorting process. Several recently completed trials indicate a significant increase in fertility; however, observed bull-to-bull variation is a major factor that needs to be ironed out. Some reports do show that in sheep, sexed semen outperforms conventional semen as far as fertility is concerned [54]. Reasons for this are varied, but the sorting process does clear out many dead and compromised sperm, and if conditions are right and timing of insemination is accurately matched with ovulation, there is a real opportunity to improve the fertilization outcome.

Summary

Despite key technological developments in recent decades, most commercial operations that utilize sorted sperm are dairy herds. Research to improve sperm sexing has been largely focused on improving sample throughput by increasing the rate at which sperm are introduced to the sorter, and on improving measurement resolution, which has increased the proportion of sperm that can be reliably measured and sorted. Although the speed of sorting has greatly increased, sperm still need to be individually assessed and sorted. As a consequence, the use of lower sperm dosage for AI has reduced fertility (compared with conventional sperm) and has contributed greatly to the limited commercial use. Furthermore, more than half of the sperm are discarded. Several improvements have recently made sperm sorting more efficient and less harmful to sperm. Modification of the technology with a harmless sorting method has great promise to improve the longevity and fertilizing capacity of sorted sperm. With further improvements in speed of sorting and precision, the cost of the process will be reduced, particularly for high-value samples. In the future, the focus may shift to other aspects of the overall process such as improvement in post-sort sperm quality to reduce bull-to-bull variations and increase the semen supply from high-demand bulls. Further, it is essential that, as commercialization increases, more data regarding fertility, embryonic losses, stillbirth, and heifer health be collected. Such information will help to evaluate the technology correctly for its practical use.

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Control of Semen-Borne Pathogens

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Introduction

Preventing the dissemination of pathogens is an essential responsibility for the bovine artificial insemination (AI) industry. Each year, millions of units of cryopreserved bovine semen are produced and distributed within the USA and around the globe. To reduce the risk of spreading disease, clear and detailed standards for production of semen from donor bulls have been established. To comply with these standards, donor bulls must complete a rigorous, prescribed testing protocol before entry is permitted into a biosecure AI center (AIC) and donor bulls must be continually monitored using prescribed testing protocols while they are producing semen. As new diagnostic tests are developed and research reveals new information regarding the epidemiology of disease transmission, these testing protocols are continually assessed and improved to ensure the absence of transmissible pathogens in semen.

In the USA, Certified Semen Services, Inc. (CSS) establishes minimum testing requirements for donor bulls entering and residing in AICs for domestic distribution of semen that is validated to be free of pathogens. Additional testing requirements may have to be met if frozen semen is intended for international export. These requirements are negotiated by the United States Department of Agriculture (USDA) and the specific importing country. Countries may establish their own requirements for testing, though these requirements are often based on guidelines published by the World Organisation for Animal Health (OIE) for the collection and processing of bovine semen. An intended purpose of the OIE guidelines is to maintain the health of animals in an AIC to allow the international distribution of semen with negligible risk of infecting people or animals with pathogens [1].

In general, donor bulls and animals intended for use as mount animals undergo testing in three phases. Selected animals are first tested prior to entering isolation (i.e. quarantine) facilities of the AIC. The second phase involves testing throughout and at the end of a quarantine period. The isolation facilities and equipment must be effectively separate from those used for the AIC resident herd. In optimal situations for quarantine, no additional animals should be added to or contacted by the distinct group of animals after completing assembly in the isolation facility. Finally, once animals have entered the resident population of the biosecure AIC, testing is maintained on an annual or semi-annual basis to monitor the population's disease-free status. Additional testing may be necessary depending on the requirements of various export markets.

This chapter covers the major diseases which – in the absence of appropriate control procedures – could be transmitted via frozen bovine semen. The pathogens involved and appropriate testing strategies to minimize the risk of disease transmission are reviewed.

Bovine Viral Diarrhea

Bovine viral diarrhea virus (BVDV) is a single-stranded RNA *Pestivirus* belonging to the Flaviviridae family. This virus has been detected throughout the world and can cause disease that significantly impacts the production and profitability of infected herds. Clinical consequences vary and include respiratory, gastrointestinal, and reproductive effects, with the major source of infection being persistently infected (PI) animals. These PI animals will efficiently transmit BVDV to susceptible animals in the herd, causing acute, transient infections that may lead to reduced

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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pregnancy rates in heifers and cows. Several studies have demonstrated that BVDV can be present in semen from bulls that are persistently or acutely infected [2–5]. These bulls can transmit BVDV through semen to susceptible recipient cattle [2–5]. In addition, bulls may exhibit testicular infections in the absence of viremia [6–8]. These localized testicular infections, which are notably rare, can result in transmission of BVDV in semen [6–8].

PI animals are a result of exposure to non-cytopathic virus through transplacental infection of the developing fetus prior to 125 days of gestation. Bulls that are PI can appear normal but are immunotolerant to the infecting strain of BVDV [9]. The PI bull is capable of shedding BVDV for long periods of time and in very high concentrations despite producing semen with normal concentration, motility, and morphology [2]. The effect of using semen from PI bulls to inseminate susceptible females is significant. One study showed that 12 of 12 seronegative and virus-negative heifers artificially inseminated with semen from a PI bull seroconverted within two weeks after exposure [3]. Another study showed that 161 of 162 cows that were inseminated with semen from a PI bull were seropositive compared with 95 of 143 cows that were inseminated with semen from other bulls, indicating that semen from PI bulls has the potential to consistently transmit BVD infection in susceptible herds [2]. This potential highlights the importance of properly screening bulls for PI status prior to entry into an AIC.

Before entry into an AIC, testing requirements to preclude BVD PI status of bulls include two negative tests (at least 10 days apart) for BVDV antigen in serum or whole blood. Because of interference of maternal antibodies with detection of circulating BVDV in serum, bulls that are less than six months of age must be tested using whole blood [10]. CSS standards require that serum or whole blood is tested using virus isolation (VI), polymerase chain reaction (PCR), or antigen capture enzyme-linked immunosorbent assay (ELISA), with the first sample collected within 30 days prior to entry into the AIC isolation facility. The bull is then sampled for a second BVDV antigen test on serum or whole blood at least 10 days after entry into the AIC isolation facility.

Susceptible bulls exposed to BVDV can become acutely infected and exhibit viremias for a period of 6–21 days after exposure. This period of viremia coincides with increasing production of antibodies specific to the infecting strain of virus [11]. Thus, increasing specific antibody concentrations indicate immune response to a recent viral exposure, while high antibody concentrations indicate prior exposure. BVDV may be detected in semen from acutely infected bulls throughout and slightly beyond the duration of viremia. Yet, concentrations of virus in semen from acutely infected bulls are significantly less than those produced by bulls that are PI [2, 7, 12]. As a result, the immediate seroconversion rates of animals inseminated with contaminated semen from acutely infected bulls (1.8–15.4%) are notably lower than those of animals inseminated with semen from PI bulls (often 100%) [3–5]. Despite the lower seroconversion rates, testing for acutely infected bulls in an AIC is important to prevent the transmission of BVDV to susceptible herds because transmission to a single naïve animal can lead to rapid amplification of virus within susceptible herds [4].

Testing requirements to prevent semen being collected and distributed from bulls that are exhibiting an acute infection with BVDV include all the testing previously described to prevent entry into AICs of PI bulls, with some additional specifications. If the result of the described test during isolation is positive, the test-positive bull is effectively isolated from other animals in the quarantine facility and tested again at least 21 days later. Other animals in the isolation facility at the time of sample resulting in the positive test must undergo additional testing. If the second result is again positive, this indicates the bull is a PI animal and is not allowed entry into the AIC resident herd. If the second test result is negative, the previous test-positive bull must be tested a third time, with the sample collected at least 10 days after the second sample. The third test must produce a negative result before the bull can enter the production herd [10]. In the event that BVDV is isolated from a bull as a result of an acute infection, all semen batches collected 30 days prior and 30 days after the blood sample that produced the positive result must be tested with VI or PCR and determined to be test-negative before they can be distributed.

Optional testing for BVDV acute infections recommended by the OIE includes a test for the detection of BVDV antibodies. Serum samples are collected prior to or at the beginning of the isolation period and at least 21 days after entry into the isolation facility. Seroconversion indicates exposure during the isolation period and the bull must be retested prior to entering the resident herd. The OIE also recommends that bulls are sampled on an annual basis and tested for BVDV antibodies to monitor for seroconversion while they are residents of the production herd of the AIC [1].

Multiple reports describe bulls with persistent testicular infections that shed infectious virus in semen despite absence of a viremic state and presence of circulating antibodies for BVDV [6–8]. This is thought to be caused by an initial infection which generates an antibody response that is unable to rapidly clear virus from within seminiferous tubules due to the blood–testes barrier [6, 7]. Reports demonstrate that bulls may consistently produce semen containing virus over an extended period of time, although the viral load in the insemination dose was less than that of

semen produced by PI bulls but more than that present in semen from acutely affected bulls [6, 8, 13]. One study reported that use of cryopreserved semen from one of these bulls resulted in seroconversion in one of three heifers that were inseminated [14].

Prolonged testicular infections (PTIs) are also reported in bulls that had detectable RNA of BVDV in the semen for up to 2.75 years following experimental acute infections [7]. However, infectious BVDV could be detected in semen using VI testing only during the viremic period of infection, despite presence of infectious virus in testicular biopsies up to 12.5 months after exposure [7, 15]. So, while VI was unable to detect infectious BVDV in semen from these bulls beyond 17 days after experimental infection, PCR detected viral RNA of neutralized virus for prolonged periods. Uniquely, one experiment demonstrated that raw semen collected from a PTI bull at five months after infection resulted in the seroconversion of a six-month-old calf when the semen was injected intravenously [15]. In another trial, repeated attempts resulted in neither seroconversions of calves inoculated intravenously with raw semen, nor seroconversions of heifers inseminated with frozen semen. It was concluded that the risk of viral transmission from PTIs with BVDV may be negligible, but additional information is required to substantiate this conclusion [7].

CSS requires that bulls housed in an AIC must be tested in one of two ways for testicular infections prior to semen release for distribution. The first method is to test all bulls in isolation with a BVDV serum neutralization test. Bulls that test antibody positive are required to have one batch code of frozen processed semen tested using VI or PCR. The second method is to require all bulls in isolation, regardless of their antibody status, to have one batch code of frozen processed semen tested using VI or PCR. Any bull found to have persistent testicular infection for BVDV is not eligible for semen distribution and is not permitted to remain in the resident production herd [10].

Leptospirosis

Leptospirosis is caused by a Gram-negative spirochete bacterium that occurs worldwide in cattle, sheep, goats, pigs, and horses. It is also a zoonotic disease and humans may acquire the infection by contact with the urine of affected animals. *Leptospira interrogans* is the pathogenic form of the bacteria and has many serovars based on cell surface antigens. Clinical signs in cattle include hemolytic anemia, drop in milk production, and changes in milk quality, stillbirths, and abortions [16, 17]. In the USA, common serovars include *L. interrogans* serovar *hardjo* (*Leptospira hardjo*), *Leptospira pomona*, *Leptospira grippotyphosa*, *Leptospira canicola*, and *Leptospira icterohaemorrhagiae*. Cattle are the reservoir host for *L. hardjo*.

Although the main route of transmission of leptospirosis is urine, studies have demonstrated that transmission of the organism may be possible through fresh and frozen semen [18, 19]. To reduce the risk of transmission through AI, the addition of antibiotics to extended semen is effective in reducing infectious leptospiral organisms that may be present in the semen without affecting semen quality [18].

In the USA, CSS requires bulls entering an AIC to be negative to a microscopic agglutination test (MAT) at 1 : 100 or demonstrate a low stable titer (negative at less than 1 : 400) when tested at least two to four weeks apart within 30 days of entering the isolation facility of the AIC for each of the following serovars: *L. hardjo*, *L. pomona*, *L. grippotyphosa*, *L. canicola*, and *L. icterohaemorrhagiae*. The same test and test results must be obtained while the bull is in the isolation facility before entering the resident production facility and the test repeated at six-month intervals while the bull is in the resident production facility of the AIC [10]. Because of the test requirements for bulls to enter an AIC, vaccination for leptospirosis is not done as the MAT is not able to distinguish between titers that are the result of natural infections and those due to vaccines.

Brucellosis

Brucella abortus is the main causative agent of brucellosis in cattle that causes abortion, retained placenta, orchitis, epididymitis, and impaired fertility. Transmission occurs primarily through contact with the uterine discharges associated with an aborted or full-term fetus, but spread through bovine milk and semen is also possible. Bulls will shed *B. abortus* organisms in semen during the acute phase of infection, after which shedding will cease or become intermittent [20]. Brucellosis as an important zoonotic disease in humans and many countries have implemented programs to eradicate *B. abortus* through vaccination and test and slaughter strategies. In the USA, the USDA has designated all 50 states brucellosis free since 2009, although infections continue to be detected in the Greater Yellowstone Area of Idaho, Montana, and Wyoming.

Several serological tests are available to detect antibodies to *B. abortus*. For bulls entering an AIC, CSS requires a negative result on a buffered *Brucella* antigen test (card or buffered acidified plate antigen [BAPA]) or a complement fixation (CF) test within 30 days prior to entry into the isolation facility of the AIC. During the isolation period, the bull is required to have a negative result on both the buffered *Brucella* antigen test (card or BAPA) and the CF test

before the bull can enter the resident production facility, and the same tests are repeated at six-month intervals while the bull is in the resident production facility. If the test results are non-negative, another approved serological test can be completed with a negative result; however, final classification of the bull is determined by the regulatory officials of the state from which the testing is completed [10].

Tuberculosis

Mycobacterium bovis is the main causative agent of tuberculosis in cattle and is a chronic progressive disease that frequently causes progressive emaciation of the affected animal. It is spread to other animals through inhalation or ingestion of *M. bovis* organisms via exhaled air, sputum, milk, urine, and feces. Transmission of tuberculosis has been reported via natural breeding from bulls with genital lesions caused by *M. bovis* and the organism has been demonstrated in fresh and frozen semen samples using PCR [21, 22]. Like brucellosis, bovine tuberculosis is a zoonotic disease and many countries have attempted to eradicate the disease through test and slaughter programs. In the USA, the USDA has designated the majority of the states as Accredited-free states, with the remainder classified as Modified Accredited Advanced states with designated prevalence rates of less than 0.01% [23].

In the USA, surveillance testing for *M. bovis* is performed on cattle by intradermal injection of bovine tuberculin in the caudal fold area at the base of the tail and observation for a reaction by the injecting, accredited veterinarian is performed 72 hours later. Animals that show a palpable response are followed up with a comparative cervical test using bovine and avian tuberculin, with measurement of the response 72 hours later. A serological test is available that measures gamma interferon response to bovine tuberculin but is limited for use only by regulatory officials.

For bulls entering an AIC, CSS requires a negative intradermal tuberculin test within 60 days prior to entry into the isolation facility. Another intradermal test is completed while the bull is in the isolation facility and a negative test is required to enter the resident production facility; the same test is repeated every six months with a negative result while the bull is a resident of the production facility of the AIC [10].

Campylobacteriosis and Trichomoniasis

Campylobacteriosis and trichomoniasis are venereal diseases in cattle that cause infertility, early embryonic death, and abortion, and, in the case of trichomoniasis, pyometra in infected females. Campylobacteriosis is caused by *Campylobacter fetus* subsp. *venerealis* which is a microaerophilic Gram-negative bacterium. Trichomoniasis is caused by *Tritrichomonas foetus*, a flagellated protozoan. Both organisms reside on the penile and preputial mucosa of infected bulls, causing no clinical signs in the male. Bulls become infected after breeding infected females. A carrier state exists for both diseases, predominantly in older bulls. Infected bulls readily transmit *C. fetus* subsp. *venerealis* and *T. foetus* by natural breeding of females. Transmission through AI is possible for both diseases [24, 25].

Diagnostic testing for *C. fetus* subsp. *venerealis* and *T. foetus* depends on the demonstration of both organisms from preputial smegma collected by aspirating or washing of the internal prepuce and penile mucosa. The preputial smegma is inoculated immediately into transport media and shipped to the diagnostic laboratory for testing. CSS requires a culture test for both organisms for each sample with a negative result. CSS does allow initial screening for *C. fetus* subsp. *venerealis* using a fluorescent antibody test, but all positive results using this test must be confirmed using bacterial culture [10]. Other diagnostic tests on preputial smegma are available including PCR and ELISA for *C. fetus* subsp. *venerealis* [26, 27] and PCR for *T. foetus*.

The prescribed frequency of testing is the same for both *C. fetus* subsp. *venerealis* and *T. foetus*. CSS requires serial testing at weekly intervals of bulls present in the isolation facility of the AIC prior to entering the resident production herd. The number of tests required in isolation depends on the age of the bull, with bulls older than one year of age requiring six tests done at weekly intervals on preputial smegma with negative results. Bulls less than one year of age require three tests on preputial smegma with negative results. While resident in the production herd, bulls are to be tested every six months for each disease with negative results [10]. The OIE recommends that bulls older than six months of age be tested three times at weekly intervals and bulls less than six months of age be tested once with a negative result while in the isolation facility and once on an annual basis when in the resident production herd [1].

The addition of antibiotics has been shown to control *C. fetus* subsp. *venerealis* organisms present in bovine semen [28, 29]. In addition to the testing requirements for *C. fetus* subsp. *venerealis*, for bulls to enter and remain in an AIC, CSS requires the addition of an antibiotic combination of gentamicin, tylosin, and lincomycin–spectinomycin to be added to raw semen and again included in the extender that is added for final dilution prior to freezing semen [10]. Acceptable antibiotic combinations recommended by the OIE for addition to extended semen include gentamicin, tylosin, lincomycin–spectinomycin; penicillin, streptomycin,

lincomycin–spectinomycin; or amikacin and dibekacin [1]. The addition of antibiotics does not control for the presence of *T. foetus* in fresh or frozen semen.

Infectious Bovine Rhinotracheitis

Infectious bovine rhinotracheitis (IBR) is a viral disease of cattle that causes upper respiratory disease, abortions, infertility, encephalitis, conjunctivitis, vulvovaginitis, and balanoposthitis. The disease is caused by bovine herpesvirus type 1 (BHV-1) and type 5 (BHV-5). BHV-1 can be further classified into two subtypes. BHV-1.1 is commonly associated with the respiratory form of the disease (IBR) and BHV-1.2 is commonly associated with the genital form of the disease causing infectious pustular vulvovaginitis (IPV) in females and infectious pustular balanoposthitis (IPB) in males. Both subtypes can cause abortions. BHV-1 is spread via aerosol transmission for the respiratory form and venereally for the genital form. Virus has been found in fresh and frozen semen and transmission through AI has been demonstrated [30]. BHV-5 is an encephalitic form of the disease that is primarily spread through aerosol transmission and has recently been reported to cause genital tract lesions and abortions. BHV-5 has been detected in semen from infected bulls and transmission through AI is possible [31].

Primary infection with BHV-1 in bulls can result in shedding of the virus from the prepuce, beginning 7–10 days after infection, and can last for several days to several weeks. After the initial phase, BHV-1 becomes latent in the infected bull and intermittent shedding can result throughout the animal's lifetime. The virus can remain latent indefinitely, although administration of corticosteroids or a period of stress such as transport can result in the recrudescence of BHV-1 and subsequent shedding of the virus from the prepuce. Contamination of semen with BHV-1 is likely the result of virus present on the mucosa of the prepuce and not from the testicles, epididymis, or accessory sex glands [30]. Although exposure leads to antibody production, the presence of antibodies may not prevent the recrudescence and shedding of latent virus in the prepuce and levels of antibodies may be very low or absent in bulls that have remained latent for a long period [19].

There are several recommendations for the testing and maintenance of bulls in an AIC. CSS does not require testing of bulls for IBR [10]. This is based on several studies showing that semen from seropositive bulls appropriately managed in AICs did not shed BHV-1 in semen [32]. This lack of shedding was attributed to the stress-free environment in which bulls reside in AICs [32]. Serologic tests are available to detect antibodies to BHV-1 and include serum neutralization and ELISA. A seronegative antibody test prior to and at least 21 days after semen production will indicate that the bull was not actively infected during the collection interval. For seropositive bulls, VI and PCR tests are available to test semen batches for presence of virus. The OIE recommends maintenance of an IBR-free herd in an AIC [1]. Bulls entering an isolation facility are to come from an IBR-free herd or undergo a serologic test for antibodies within 28 days prior to entry with a negative result. The bull is to be tested in isolation with a serologic test with a negative result and all the bulls in the isolation facility must be negative on a serologic test prior to entry into the production herd. The production herd is tested with a serologic test on an annual basis and all resident bulls are required to produce a negative result [1].

Arboviruses

Cattle are susceptible to arthropod-borne viruses that have the potential to be transmitted through semen. Cattle may become infected via insects that harbor the virus after feeding on viremic animals and subsequently feeding on susceptible animals, thus transmitting the virus. Distribution of disease is limited to the endemic areas where the insect vectors reside, and viruses are often maintained in these areas through wildlife reservoirs. New epidemic areas occur through the movements of virus-infected vectors or movements of viremic host animals into new areas with competent vectors.

Bluetongue virus (BTV) and epizootic hemorrhagic disease (EHD) virus are orbiviruses that have worldwide distribution. There are at least 27 distinct serotypes of BTV and the virus is mainly transmitted by midges (*Culicoides* species). Cattle are a major reservoir host for the virus. Infected cattle rarely show clinical symptoms, but the disease may infrequently cause fever, facial edema, hemorrhages, and ulceration of the mucous membranes in some cattle. BTV has been found in semen but only consistently during the viremic period of infection in bulls, possibly associated with blood that has leaked from the genital tract [9].

EHD is similar to BTV and there are at least seven serotypes, with significant immunological cross-reactivity between the two viruses. EHD is also similar to BTV in that it rarely causes clinical signs in cattle and is mainly transmitted by midges (*Culicoides* species). The presence of EHD in semen has not been demonstrated, but, due to its similarity to BTV, international recommendations include testing for the virus in bulls used for AI [1].

Serologic and virus identification tests are available for monitoring bulls and semen used for AI. Serologic tests that are available include agar gel immunodiffu-

sion and ELISA. The OIE recommends that seronegative bulls are tested prior to semen collection and again 21–60 days after collection with negative results. Virus identification tests include VI and PCR done on whole blood. The OIE recommends either VI or PCR prior to collection and again at least every 7 days during the collection period for VI or at least every 28 days during the collection period for PCR and a final test at the end of the collection period [33]. VI and PCR tests are also available for frozen semen.

Recently, a novel arboviral disease emerged in Europe caused by Schmallenberg virus (SBV). Disease was first detected in November 2011 in dairy cattle that had reduced milk production and diarrhea. The virus was later found to cause congenital malformations in aborted and newborn calves. SBV is an *Orthobunyavirus* and is a member of the Simbu serogroup and is transmitted by *Culicoides* midges. The virus has been detected in bovine semen by PCR and is able to be shed in consecutive semen batches or intermittently for 43 days in one report [34] and as long as three months in another report [35]. A single insemination dose of semen can contain SBV sufficient to infect naïve cattle through experimental subcutaneous injection, though transmission of SBV by natural breeding or AI remains to be demonstrated [36]. Serological tests are available including serum neutralization and ELISA, while PCR and VI are available to detect virus antigen in blood. To validate semen free of SBV, testing of semen samples for SBV RNA using PCR is recommended unless the bull tests negative for SBV-specific antibody at least 28 days after semen collection [37].

Enzootic Bovine Leukosis

Enzootic bovine leukosis is a retroviral disease of cattle that causes persistent lymphocytosis and lymphosarcoma in affected cattle and is caused by bovine leukemia virus (BLV). BLV is found in lymphocytes and can be transmitted horizontally via contact with secretions from infected animals, iatrogenically via contaminated instruments such as tattoo pliers and dehorning gouges, and vertically via transplacental exposure.

There has been one study that reported the presence of BLV in bull semen by demonstrating seroconversion of sheep that were injected intraperitoneally with semen collected from a BLV-positive bull [38]. The semen from this bull was collected by rectal massage, which may have resulted in excess trauma and presence of lymphocytes in the semen. A follow- up study repeated the experiment using 32 seropositive bulls that were collected normally and no seroconversions were reported in inoculated sheep [39]. A study following the use of semen from BLV-seropositive bulls collected and processed in an AIC showed no evidence of seroconversion of females in a BLV-free herd over a five-year period when bred with AI [40]. Another study tested frozen semen batches from BLV-seropositive bulls using PCR and did not find any evidence of BLV [41]. Semen collected in these studies were from bulls residing in AICs where semen was collected using an artificial vagina and routine laboratory procedures used to evaluate the semen for quality to exclude the presence of white blood cells.

Serologic tests for BLV, including agar gel immunodiffusion and ELISA, are available to accommodate international requirements for trade in bovine semen. The OIE recommends either that donor bulls reside in a BLV-free herd or serologic testing of the bull at least 30 days before and at least 90 days after the collection of semen. In the USA, CSS does not require testing of donor bulls for leukosis for residence in a production herd [10]. VI and PCR tests are available to detect virus antigen in the blood and a PCR test is available to test semen.

Control

Control of pathogens in bovine semen, especially from bulls residing in AICs, is primarily dependent on frequent testing to demonstrate lack of detection of pathogens of concern as well as antibody responses to the specific pathogens. Several diseases such as BVDV and leptospirosis require the resident production herd to maintain freedom from infection. A positive test result generally requires the removal of the positive animal and retesting of the resident herd population. Other diseases such as BTV and EHD allow seropositive animals to be present in the resident herd and tested differently to minimize the risk of the pathogen being present in semen. Seronegative animals are tested for antibodies before collection of semen and again after the collection to demonstrate lack of exposure to the pathogen. Testing is done on seropositive animals using tests to detect the pathogen during the time of collection in semen or blood. The testing strategy for each AIC will depend on specific regulations and standards of the country where the AIC is located, and standards required for export of cryopreserved semen to foreign markets.

Minimizing the introduction of diseases is an important consideration for maintaining a disease-free herd. Biosecurity protocols are necessary to reduce the probability of introduction of disease. Bulls should be housed indoors or in paddocks with perimeter fencing to prevent contact with other livestock and wildlife. Barn personnel should change into clothing dedicated to the facility and, at a minimum, should wash their boots prior to entry into the facilities and when moving between quarantine and

isolation facilities. Outside vehicles should be clean and have tires disinfected prior to entering the AIC. Bulls should be maintained in a clean environment and not soiled during collection. Bulls should be maintained in good health and physical status. Sick animals should be isolated and not used to collect semen. Many countries require that minimum standards for biosecurity are maintained for semen to be released for distribution both domestically and internationally. Biosecurity and standard operating practice manuals should be present and frequently updated and followed to reduce the potential for introduction of disease into an AIC.

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Bovine Semen Quality Control in Artificial Insemination Centers

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Introduction

Fertility is a multiparametric phenomenon that relies on the use of semen of sufficient quality and quantity, accurate timing and method of insemination, and appropriate herd management. When using artificial insemination (AI), the dairy producer must manage a range of these factors, including heat detection, timing of insemination in relation to estrus, and correct handling of the frozen straws. However, it is the onus of the semen production centers (SPCs) to supply straws containing spermatozoa of good viability that produce acceptable conception rates if all other variables are managed correctly.

To ensure acceptable fertility after AI, frozen–thawed spermatozoa must be present in sufficient number in each straw (concentration) and possess a number of characteristics important for fertilization. Accordingly, spermatozoa must survive the thawing procedure with normal morphology, an intact acrosome, DNA integrity, and active mitochondria, and maintain forward progressive motility to traverse the female reproductive tract. Some or many of these characteristics are measured during post-thaw quality control (QC) procedures undertaken by SPCs prior to distribution. QC is the assurance that each batch of straws has undergone semen analysis to verify that the sample is likely to be fertile.

Although semen analysis may seem easy to perform, meticulous attention to detail and technique is essential in order to obtain an accurate and reproducible analysis. Manual semen analysis using a light microscope has been the standard method for analysis in most SPCs. However, manual analyses can be very subjective and prone to within- and between-technician errors. Similarly, the use of fluorescence microscopy to assess spermatozoa for acrosome, membrane, and DNA integrity is markedly slow and limited due to the low number of spermatozoa analyzed from each sample and the incapacity for an extensive multiparametric analysis.

To maximize accuracy in QC, SPCs are realizing the benefit of a multiparametric approach and have increased the rigor of their semen testing, moving from time-consuming basic subjective assessment of a few hundred spermatozoa for concentration, motility, and morphology using microscopy, to the use of computer-assisted tracking to assess motility and flow cytometry to analyze thousands of cells within seconds for characteristics such as viability, mitochondrial activity, acrosome, DNA and capacitation status. The topics for discussion in this review are the various tools and assays in use in cattle SPCs to determine QC values, factors to consider when using these tools, and how the efficacy of QC procedures may be maximized in order to predict field fertility.

Objective Assessment of Sperm Motility

Computer-assisted sperm analysis (CASA) is a powerful tool for the objective assessment of sperm motility and is hence now frequently used for evaluating semen quality. The basic components of this technology consist of a microscope to visualize the sample, a digital camera to capture images, and a computer with specialized software to analyze the movement of the spermatozoa. The essential principle behind most microscopy-based CASA systems is that a series of successive images of motile spermatozoa

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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within a static field of view are acquired by computer software algorithms, which then scan these image sequences to identify individual spermatozoa and trace their progression across the field of view. This involves recognizing the same cell in each image by its position and inferring its next position by estimating the likelihood that it will only have moved a certain maximum distance between frames. CASA can also provide information about sperm concentration, morphology, viability, and index of DNA fragmentation of frozen–thawed sperm. However, these more specialized techniques are not routinely applied for regular analysis of frozen–thawed semen in SPCs.

With the use of CASA, several motility parameters describing the specific movements of spermatozoa can be obtained in greater detail than possible in subjective assessment. These computerized measurements can be useful for assessing various sperm characteristics simultaneously and objectively, and are valuable for the detection of subtle changes in sperm motion that cannot be identified by conventional subjective semen analysis, as reviewed elsewhere [1–3]. The parameters typically collected using CASA systems are motility, velocity, linearity, and lateral displacement of spermatozoa as they progress along their trajectories in a sample. The percentages of total and progressive motility are the most important motility parameters in the evaluation of spermatozoa. Total motility refers to the fraction of spermatozoa that display any type of movement, whereas progressively motile spermatozoa swim forward in an essentially straight line. Spermatozoa that swim but in an abnormal way, such as in tight circles, are not included in the proportion of progressively motile sperm. In addition to evaluation of sperm motility, the software calculates the kinetic values of each spermatozoon, which covers the velocity of movement, the width of the sperm head's trajectory, and frequency of the change in direction of the sperm head. The velocity values that are determined by CASA are the curvilinear velocity (VCL), straight-line velocity, and average path velocity (VAP) [2]. The amplitude of lateral head displacement and beat cross frequency (BCF) are two other characteristics measured with CASA instruments [2].

Limitations of CASA Instruments

Despite the power of an objective evaluation by CASA, there are some constraints associated with this technology. Many factors are known to affect CASA results. The type of specimen chamber used for analysis can affect the movement of sperm, the accuracy of the cell count number, and therefore the percentage of motile spermatozoa [4]. The temperature at which semen is analyzed is also an important factor that may affect CASA results. Independent studies showed that analyzing semen below 37 °C significantly affected results [5, 6]. These groups performed CASA on spermatozoa maintained at 37 °C with a stage warmer and compared the results with spermatozoa analyzed at room temperature or at 30 °C. The data demonstrated a decrease in the motility parameters (percentage of motile spermatozoa and track speed) when spermatozoa were not analyzed at 37 °C. These experiments suggest that a simple variation introduced in the analysis of sperm motility can have a considerable effect on the results. The concentration at which semen is analyzed is an essential aspect that influences CASA results. It has been established that at low semen concentrations (<20 million/ml), an overestimation of the concentration and thus an underestimation of the percentage of motile cells can occur due to the acquisition of non-spermatic particles (debris). On the other hand, at higher concentrations (>50 million/ml), a large proportion of the fast-moving cells will be excluded from analysis because of cell collisions or spermatozoa exiting the analysis area, or excluded on the basis of nearest-neighbor effects, leading to an underestimation of the motility [5, 7].

Sampling condition is a source of error when acquiring data with CASA. Computer and video camera equipment are continuously evolving and different CASA systems use various models of video camera. Most of the CASA systems allow 30 or 60 Hz as a frame rate to analyze sperm tracks and speed. Studies have shown the importance of the frame rate for reliability of the analysis [8–10]. It is generally accepted that a higher frame rate is required to render an evaluation closer to the real path for a fast non-linear sperm cell. To study a hyperactivated sperm cell, Mortimer and Swan [11] suggested using the highest frame rate available on the system in order to have the most accurate evaluation.

The type of extender in which semen is diluted is another aspect that should be taken into consideration when evaluating spermatozoa with CASA. Some extenders contain debris of a size similar to a sperm head, causing CASA software to include them in the analysis. Egg yolk- and milk-based diluents are examples of extenders containing such particles. In addition, when observing semen diluted with milk extender, the globular lipids mask the spermatozoa, thus rendering CASA analysis impossible. To assess motility analysis in these conditions, samples could be washed to remove extender debris from semen. However, it has been established that washing the semen affects the motility of the spermatozoa [12], making correct evaluation more difficult. To overcome this problem, fluorescence technology allows discrimination of sperm cells from particles in the extender by staining sperm heads with a

DNA-binding fluorochrome. Under fluorescent light, only DNA-containing objects will be detected by the CASA software, thus omitting the need for washes. This technique improves the accuracy of the concentration [13] as well as the motility analysis [14] when working with semen diluted in these extenders. Therefore, standardizing the type of chamber, the temperature, the concentration, and the type of extender is crucial to assure repeatable standard QC at an SPC.

Motility is one of the most important characteristics believed to be associated with the fertilizing ability of spermatozoa. Several groups have reported a significant correlation between total [15–18] and progressive [19] motility of bull semen and its associated field fertility. However, other groups have reported that the subjective analysis of semen motility did not correlate with fertility [20, 21]. CASA instruments collect a wide range of sperm motility parameters, allowing a more detailed and accurate analysis of sperm movements and track speed. Researchers have also tried to correlate the kinetic parameters with the field fertility of semen, with some groups able to show a positive correlation between straight-line velocity of spermatozoa and field fertility [18–22]. Another study used a combination of several motility parameters to reach a very high correlation with bull fertility [20]. Taken together, these studies show the high potential of CASA for estimating the quality of the semen and therefore becoming a powerful tool to measure sperm characteristics and predict bull fertility compared with standard semen evaluation. However, as mentioned above, standardization of conditions and parameters of all CASA analyses is key to obtain repeatable and valid correlations with fertility.

Several models of CASA instruments are now available to evaluate the quality and motility of spermatozoa. Each system operates on similar principles but they differ in their parameter settings and use different algorithms to determine speed and trajectories. Parameter settings, threshold settings, video frame rate, and other variables will affect CASA results, as reviewed by Davis and Katz [23]. As mentioned above, new technologies and CASA software evolve quickly. Our laboratory undertook a small study to measure the aptitude of the CEROS™ (Hamilton-Thorne, USA) and the Sperm Class Analyzer® (SCA; Microptics, Spain) in evaluating the motility and the concentration of frozen–thawed bovine spermatozoa diluted in an egg yolk-based extender (unpublished data). A total of 18 different frozen–thawed ejaculates were analyzed with both systems and the mean total and progressive motility percentages, concentration, VAP, VCL, straight-line velocity, BCF, and amplitude of lateral head displacement were compared (Figure 81.1). Among all parameters analyzed, only the percentage of total motile cells was not significantly different between the systems. The discrepancies can be explained by the better capacity of the SCA to exclude egg yolk particles from the analysis. The SCA discriminated non-spermatic particles based on size in microns, while the CEROS used pixels to estimate the size of the cells. Differences in the algorithms used to calculate slow, medium, and fast spermatozoa may also explain the variation in the motility and kinetic parameters observed between each system. Overall, this mini-study indicates high variability between CEROS and SCA systems in estimating sperm motility parameters.

Analysis of Sperm Function by Flow Cytometry

Flow cytometry analyzes cells suspended in a stream of fluid passing at high velocity in front of one or several lasers. The light emitted by fluorochrome-bound cells is captured by photomultiplier tubes and converted into an electronic signal, subsequently digitalized by cytometry software. Key features of flow cytometry are the acquisition and analysis of thousands of cells within seconds and the multiparametric potential of the technology. The most modern cytometers are routinely equipped with three lasers and at least 10 photomultiplier tubes, allowing cell labeling with several probes at the same time and thus enabling analysis of numerous parameters simultaneously. In the last few years, the multiparametric aspect of flow cytometry has allowed this technology to become a popular tool for evaluating sperm attributes [24–26]. A wide range of fluorochromes has been developed to assess numerous characteristics of sperm cells. Here we review some of the fluorochromes used to study sperm cells with flow cytometry.

Sperm Attributes Analyzed by Flow Cytometry

Viability/Mortality

Propidium iodide is the most popular dye used to identify dead cells. This membrane-permeable fluorochrome enters spermatozoa with damaged cellular membranes and binds to DNA, where it can be excited with a 488-nm laser present on most cytometers [27–29]. Propidium iodide is often used in combination with SYBR-14, another DNA-labeling probe [30, 31]. SYBR-14 is also excited by the 488-nm laser and is a permeant probe staining all cells. Added to the cells simultaneously, propidium iodide displaces or quenches the SYBR-14 fluorescence in damaged cells.

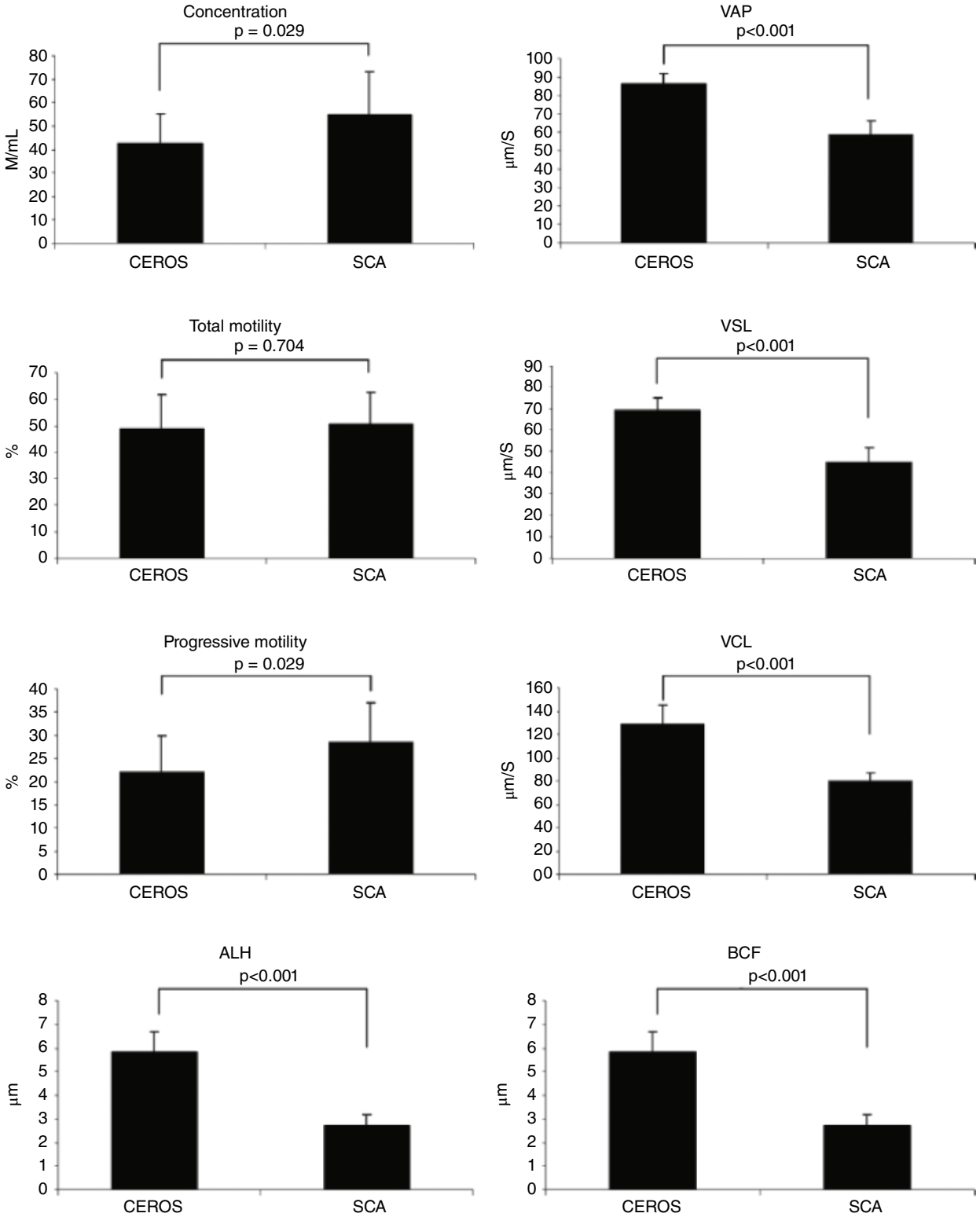


Figure 81.1 Comparison of CEROS (Hamilton-Thorne) and SCA (Sperm Class Analyzer) for determining concentration, percentage of total and progressive motility, lateral head displacement (ALH), average path velocity (VAP), straight line velocity (VSL), VCL, and beat cross frequency (BCF) from 18 different frozen–thawed bovine ejaculates. Columns represent mean values ± SEM. *P*-value <0.05 indicates a statistically significant difference between CEROS and SCA, Student's paired *t*-test.

A new fixable dye commercialized by Invitrogen under the name Live/Dead® fixable dead cell kit is now available to evaluate the viability of cells [32]. This dye reacts with cellular amines on the surface of cells or inside the cytoplasm of cells with damaged membranes. Cell-surface staining of amines of viable cells will result in relatively dim staining compared with the bright staining of dead cells. This fixable dye belongs to a large family available in different wavelengths of excitation/emission, allowing its use on most cytometers.

Acrosome Integrity

Evaluation of acrosomal status is mainly assessed by using plant lectins recognizing acrosomal ligands. *Pisum sativum* agglutinin binds mannose and galactose moieties of the acrosomal matrix. As *P. sativum* agglutinin cannot penetrate the intact acrosomal membrane, only spermatozoa with a reacted or damaged acrosome will be stained [21, 33, 34]. However, it has been shown that *P. sativum* agglutinin has an affinity for egg yolk and non-specific binding sites on the sperm cell surface [35, 36]. This aspect could become a problem when analyzing semen frozen in egg yolk-based extender and result in misinterpretation of the acrosomal status of sperm. *Arachis hypogaea* (peanut) agglutinin binds galactose moieties of the outer acrosome membrane and is the most popular lectin used to study the integrity of the acrosomal membrane with flow cytometry [37–39]. In addition, *A. hypogaea* agglutinin seems the most reliable probe for identifying spermatozoa with a damaged acrosome as it displays less non-specific binding to other areas of spermatozoa [40]. *P. sativum* agglutinin and *A. hypogaea* agglutinin are usually labeled with fluorescein isothiocyanate (FITC) fluorochromes, allowing them to be used by all cytometers.

Mitochondrial Activity

Mitochondria are very important organelles involved primarily in the generation of the energetic substrates for the motility of the sperm cell. Rhodamine 123 was one of the first probes to monitor mitochondrial activity [41, 42]. Rhodamine 123 is sequestered in active mitochondria and washed out from the cell when the membrane potential is lost. This characteristic limits its use when quantification is needed or when fixation of spermatozoa is required. To overcome the fixation problem, Mitotracker® dye could become a solution. This fixable dye accumulates and stains active mitochondria and has the advantage of availability in different ranges of excitation and emission fluorescence [42–44]. The most popular probe for evaluating mitochondrial activity is JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide) [45–47]. In spermatozoa containing mitochondria with a high membrane potential, JC-1 enters the mitochondrial matrix where it accumulates and forms J-aggregates and become fluorescent red. In spermatozoa containing mitochondria with low membrane potential, JC-1 cannot accumulate within the mitochondria and remains in the cytoplasm in a green fluorescent monomeric form. JC-1 has the advantage of being able to quantify the mitochondrial burst of the cell compared with Rhodamine 123 and Mitotracker. A disadvantage of the JC-1 probe is its dual fluorescence emission that limits its combination with other probes emitting at the green and red wavelngths.

DNA Integrity

Assessment of chromatin status is important in determination of the fertility potential of spermatozoa. In recent years, the sperm chromatin structure assay developed by Evenson and Jost [48] is the main technique used to evaluate chromatin integrity in spermatozoa by flow cytometry [49, 50]. The sperm chromatin structure assay uses the dual fluorescence emission of acridine orange, depending on whether it binds to single-stranded DNA (red fluorescence) or double-stranded DNA (green fluorescence). Following a denaturation step, the sperm sample is incubated with acridine orange and then analyzed by flow cytometry. Denaturation will induce single-strand DNA formation when DNA breaks are present and generate a heterogeneous population of red and green fluorescence depending on the integrity of the chromatin. The most important result derived from the sperm chromatin structure assay is the ratio of red/green plus red fluorescence called the DNA fragmentation index, where a high DNA fragmentation index correlates with high DNA damage. The DNA fragmentation index has shown correlation with fertility in different species [51–53]. The large luminal spectrum covered by acridine orange and the denaturation step required to induce single-strand DNA are two main inconveniences of the sperm chromatin structure assay for a multiparametric analysis. Acridine orange fluoresces in the green and red spectrum; that leaves few possibilities for adding other fluorochromes in these spectral areas and the denaturation step is performed with an acid/detergent solution not compatible with all probes. Another assay to assess DNA integrity developed for flow cytometry is the terminal transferase dUTP nick end labeling (TUNEL) assay, which can identify DNA strand breaks [54–56]. Transferase enzyme incorporates fluorescently modified nucleotides at the sites of DNA breakage. The resultant labeled cells can then be analyzed by flow cytometry. The TUNEL assay allows quantification of

labeled nucleotides incorporated into fragmented DNA reflected by the increase in fluorescence, which gives an appreciable advantage over the sperm chromatin structure assay.

Calcium Influx

Calcium influx is one of the primary steps involved in the sperm capacitation process. The rise in intracellular calcium ultimately leads to the phosphorylation of tyrosine and serine residues in proteins regulating the signaling cascade. The most popular dye used to determine the intracellular calcium concentration in sperm cells is the Fluo-3/4 family probe excited by the 488-nm laser line [57–59]. Calcium-unbound Fluo-3 is a non-fluorescent molecule, but when calcium ions enter the cell and bind Fluo-3 the latter becomes fluorescent. Fluo-4 is a derivative of Fluo-3, bearing higher fluorescence intensity. Fura red is a probe also excited by the 488-nm laser whose fluorescence emission decreases on calcium binding. Dual labeling of spermatozoa with Fluo-3/Fura red allows calculation of the ratio of unbound to bound calcium. The ratio between the two mean fluorescence intensities (Fluo-3/Fura red) is proportional to the intracellular calcium concentration of the spermatozoa. This experimental approach has been used to assess dog semen by Peña et al. [60]. One critical aspect when using these dyes is cell loading. Because mean fluorescence intensity is the parameter used to indicate the level of intracellular calcium, errors in pipetting of the probe will change the mean intensity and result in misinterpretation of the results. The drawback of Fluo-3/Fura red combination is the need for two different fluorescence detectors, which decreases the scope of a multiparametric approach. Indo-1 pentaacetoxymethyl ester is a membrane-permeable calcium sensor dye used to monitor changes in intracellular calcium. Once Indo-1 enters the cell, esterases cleave the acetoxymethyl group, yielding a membrane-permeable dye. Unbound Indo-1 has peak emission at 485 nm. On binding calcium, the peak emission shifts down to 410 nm. Measurement over time can be represented as a ratio of the two emission wavelengths. As Indo-1 acetoxymethyl is a ratiometric probe, cell-loading concerns (as for Fluo-3/4) are less important. One restriction with this probe is that not all laboratories are equipped with an instrument comprising the ultraviolet laser needed to excite Indo-1 acetoxymethyl. For those with this instrument, Indo-1 acetoxymethyl becomes a very good probe for a multiparametric approach because it is one among few probes using the ultraviolet laser, thus leaving the 488- and 633-nm lasers available to study other parameters.

Limitations of Flow Cytometry

Several factors influence the choice of cytometer for the analysis of sperm cells. The price of the instrument remains a major factor that will influence this choice. Multiparametric analysis is usually obtained with instruments containing more than one laser and many photomultiplier tubes, which increases the price of the equipment substantially. Indeed, the type of analyses to be performed is also a factor that will determine the choice of flow cytometer. Depending on the objectives of the breeding center and the experimental design, the combination of lasers (number and wavelength) and the number of photomultiplier tubes included in the instrument must be taken into account. An instrument with only one laser and three photomultiplier tubes allows detection of a maximum of three parameters on each cell, while a multiparametric analysis including four or more parameters will usually require an instrument having at least two lasers and four photomultiplier tubes. The software operating the flow cytometer is another important aspect in the choice of instrument. Most software products available are fairly easy and straightforward to operate for a novice user in flow cytometry. However, some software requires certain knowledge of flow cytometry concepts, making the instrument more difficult to operate. As an example, samples stained with a cocktail containing several probes are subjected to subpopulation gating analysis. In order to obtain representative results, gates need to be associated to the proper population in the correct hierarchy, a perspective that is difficult to handle with some software for a novice user. Moreover, some programs have gaps in export and data compilation, making it more difficult to analyze the data, and these shortcomings are time-consuming for the user.

As mentioned in the section on "Analysis of Sperm Function by Flow Cytometry," flow cytometry is a relatively new avenue for the SPCs. The unique characteristics of spermatozoa must be considered when selecting an instrument. The paddle shape of the head and presence of the flagellum make spermatozoa very different in size and cellular complexity compared with most cells studied by flow cytometry. Indeed, the majority of cells studied with this technology have a round shape, and passage in front of the laser leads to a neat forward scatter versus side scatter plot. However, when a sperm cell hits the laser, it could be on the thick or the thin side of the head. This unique feature of sperm cells will lead to a scatter plot of different size and complexity. A very important aspect we found when studying frozen–thawed spermatozoa with flow cytometry, and which has been discussed in this chapter, is the extender in which semen is diluted. As stated, different types of extenders are used to dilute semen, and some contain particles of

a similar size to spermatozoa. This aspect of particle contamination of the target population is a concern when considering the purchase of a cytometer for multiparametric analyses. An apparatus powerful enough to discriminate sperm cells from particles based only on the size and complexity of the cells allows gating of the sperm cell population without using any fluorochromes. As a result, more photomultiplier tubes are left available for cell characterization with fluorochromes. Our laboratory has compared several flow cytometers, and only half of them were sensitive enough to accomplish this discrimination between foreign particles and spermatozoa without having to use any fluorochromes. A research laboratory possessing a cytometer that cannot discriminate debris from spermatozoa will have to either wash the cells to remove particles or stain the sperm cells with a fluorochrome, which leaves fewer photomultiplier tubes to study other parameters. Not all fluorochromes are suitable with semen extenders, especially egg yolk-derived extenders known to quench some fluorescent dyes. Hoechst 33342 is routinely used to stain the nucleic acid of spermatozoa when using a cytometer equipped with an ultraviolet or violet laser. This approach allows elimination of the particles by gating them out, resulting in a more accurate analysis (Figure 81.2). Unfortunately, not all systems are equipped with such lasers to use Hoechst 33342 as a cell tracking dye, but a cell permeant dye like SYBR-14 could be added to sperm cells and this fluorescent population gated for further analysis.

A Multiparametric Approach for Standardization and QC among SPCs

Use of CASA and Flow Cytometry as QC Tools

Subjective evaluation of semen by conventional microscopy is still used by numerous SPCs. As discussed in the Introduction, this type of semen evaluation results in variation of the final decision regarding whether the semen lot will be accepted or rejected during QC. Even though CASA is used in some SPCs, fertility is a multiparametric phenomenon; thus motility parameters of spermatozoa are not sufficient for evaluating the global fertility potential of a semen sample. Introduction of flow cytometry in SPCs will allow better characterization of the spermatozoa because, by itself, flow cytometry has the potential for multiparametric analysis of spermatozoa. Combining CASA and cytometry will provide SPCs with a powerful multiparametric approach to evaluate the quality of the semen produced and allow the establishment of standardized procedures to make accurate and repeatable decisions on the outcome of the semen.

We have evaluated the potential of these tools to accurately estimate the quality of semen produced in SPCs across Canada. The percentage of total and progressive motility and the percentage of viability, acrosome integrity, and high mitochondrial activity were evaluated with CASA and flow cytometry. We obtained and reanalyzed 660 lots of semen diluted in egg yolk produced by different SPCs that had previously been processed by standard subjective QC, where 58% ($n = 385$) were accepted and 42% ($n = 275$) were rejected. Semen evaluation was performed at two different time points: immediately after thawing and two hours later after a thermoresistance stress. Cutoff values (Table 81.1) were established for each parameter analyzed with CASA/flow cytometry at each time point. Semen lots that did not reach our cutoff standard would have been rejected and discarded. On the other hand, if the quality of the semen was good and met our cutoff values, the lot was considered as accepted using CASA and flow cytometry and would have been distributed.

Table 81.2 highlights the percentage of lots rejected after analyzing the 660 lots with CASA and flow cytometry. CASA analyses revealed that 14% (94 of 660) unique lots failed to meet the cutoff immediately after thawing compared with 27% (178 of 660) unique lots after two hours' incubation. The same observation could be made from flow cytometry analyses, where 24% (156 of 660) unique lots were rejected after thawing compared with 32% (214 of 660) unique lots after two hours' incubation. This analysis demonstrates the need for a thermoresistance test to be included in QC to increase precision where the majority of lots were discarded based on the two-hour incubation. In addition, CASA evaluation alone resulted in the discard of 28% of the lots while flow cytometry analysis alone discarded 34% (224 of 660) of the lots. When CASA and flow cytometry are used in combination, a total of 41% (268 of 660) unique lots were rejected, resulting in an increase in rejection of 13% compared with CASA alone (28%). Overall, these results demonstrate the ability of the multiparametric approach to provide very high authority to the rejection/acceptance decision.

Detailed analyses of accepted and rejected lots with CASA/flow cytometry compared with standard QC showed 77.4% agreement in the decision made using the two different methods of QC assessment, but 22.6% of the samples were discordant between them (Figure 81.3). This discrepancy would be considered the "precision impact" of the multiparametric technique over the standard subjective evaluation. This 22.6% consists of 10.8% of the accepted lots by standard methods that would have been rejected with our multiparametric tools and 11.8% of the rejected lots by standard methods that would have been accepted with the multiparametric approach. At first sight, 22.6% of

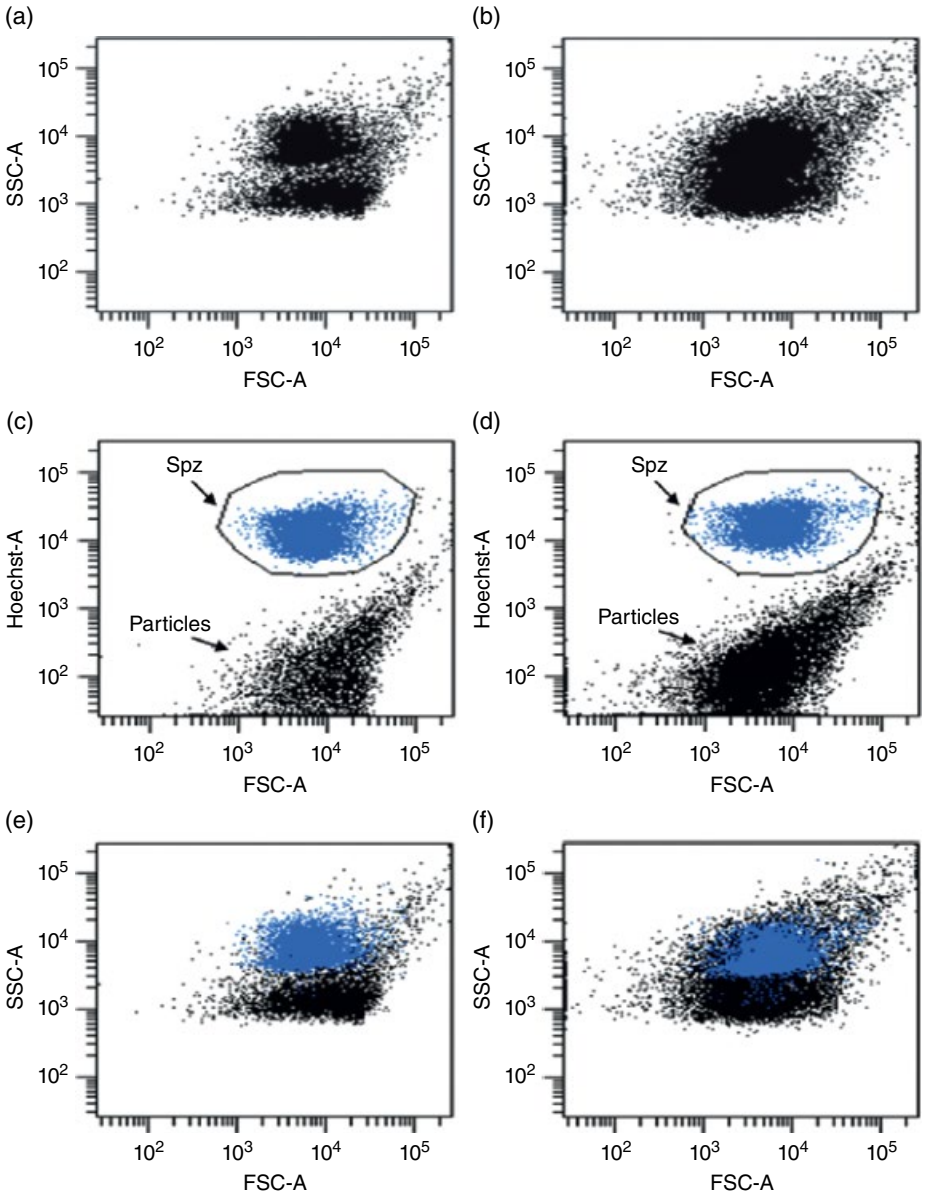


Figure 81.2 Comparison of (a) good and (b) bad resolution of spermatozoa from non-spermatic particles in frozen–thawed ejaculates. Labeling cells with Hoechst 33342 (blue cells in c, d) allows exclusive staining of spermatozoa and discrimination from cellular debris and egg yolk-derived non-spermatic particles. The positive population can be used for further characterizations in a multiparametric approach (e, f). Spz, Spermatozoa; SSC, side scatter plot; FSC, forward scatter plot.

discrepancies between objective and subjective evaluation would appear high. However, CASA/flow cytometry would have discarded 268 lots (Table 81.2) while accepting 392 lots compared with the original 385 accepted lots and 275 rejected by the SPCs. This represents a 1% difference of semen production and would be considered as the "production impact" of CASA/flow cytometry over standard QC. This study demonstrates that the production impact would be negligible but the precision impact would be quite considerable.

To estimate the impact of this multiparametric approach on semen fertility in the field, we applied this analysis on semen lots of known fertility that were released in the field after using standard QC. CASA and flow cytometry were performed on 192 lots with at least 250 first inseminations. The fertility associated to each lot was obtained from the Canadian Dairy Network and was expressed as FERTSOL, which represents the 56-day non-return rate, adjusted for multiple parameters including season of insemination, inseminator, and number of inseminations. Using our

Table 81.1 CASA/flow cytometry cutoff values (%) used to determine pass/fail rates during QC of frozen–thawed semen immediately after thawing and after two hours' thermoresistant stress. Semen lots meeting or exceeding these cutoff values passed the evaluation.

Parameter	Post thaw	After 2 hours' stress
Total motility	40	35
Progressive motility	15	10
Intact acrosome	66	61
Membrane intact cells	40	40
Mitochondrial activity	40	45

Table 81.2 Of 660 total (first number), number (%, second number in parentheses) of samples rejected for at least one QC parameter based on CASA alone, flow cytometry alone, or CASA plus flow cytometry.

Parameter	Post-thaw (0 hours)	After 2 hours' stress	0 hours + 2 hours[a]
CASA alone			
Total motility	76 (12)	157 (24)	163 (25)
Progressive motility	70 (11)	137 (21)	151 (23)
Total CASA	94 (14)	178 (27)	188 (28)
Flow cytometry alone			
Intact acrosome	104 (16)	97 (15)	117 (18)
Membrane intact cells	132 (20)	144 (22)	159 (24)
Mitochondrial activity	133 (20)	208 (32)	212 (32)
Total flow cytometry	156 (24)	214 (32)	224 (34)
Total CASA+ flow cytometry	176 (27)	260 (39)	268 (41)

[a] Unique lots rejected at 0 hours + unique lots rejected at 2 hours.

multiparametric approach, 9.4% (18 of 192) of the semen lots would have been discarded before distribution. These lots corresponded to 5.3% of the low-fertility semen (<−1 FERTSOL), 3.6% of average fertility semen (between −1 and +1 FERTSOL), and 0.5% of high-fertility semen (>+1 FERTSOL). Again, this multiparametric approach would have increased the overall fertility of semen released in the field.

Specific Applications of the Multiparametric Approach in Semen Evaluation

The multifactorial aspect of fertility can result in misevaluation of semen quality when assessing only a few parameters. Even semen that passes rigorous QC steps may have

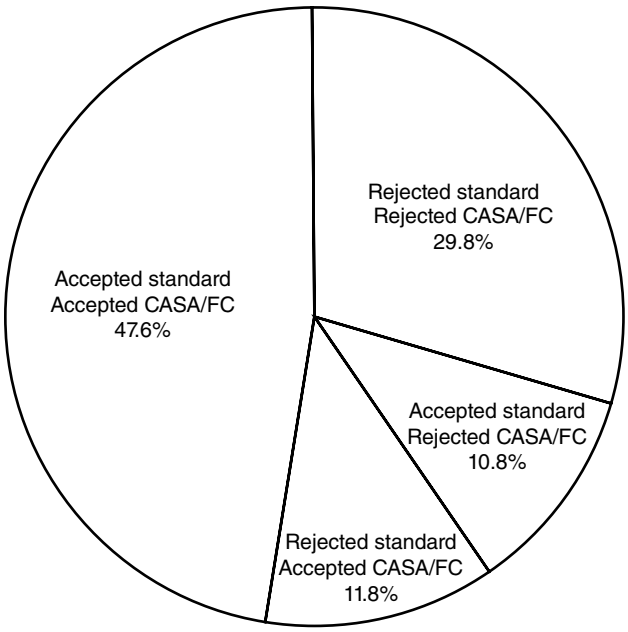


Figure 81.3 Comparative analysis of pass/fail percentage between standard QC and CASA/flow cytometry during QC of 660 frozen–thawed semen lots.

low fertility once distributed in the field. Such lots would have passed all our CASA/flow cytometry cutoff values but still resulted in few calves despite the high number of inseminations. A more detailed analysis of this semen is then required to understand its low fertility. However, deeper analyses of semen samples are time-consuming and expensive, so they are performed only on high-value semen. Here, we report two cases where flow cytometry helped us to target a putative cause of low fertility-associated semen.

The first case involved a young bull (bull A), one of whose semen lots produced very few embryos after *in vitro;* fertilization compared with the average embryo production. This lot of semen was compared (in triplicate) with one from an average bull (bull C) used as an internal control for most of our experiments. Motility and viability parameters estimated with CASA/flow cytometry showed average quality spermatozoa from both bulls (average of 36% total motility and 15% progressive motility). Evaluation of the level of intracellular calcium and the integrity of the acrosome membrane was carried out immediately after thawing and after five hours of post-thaw incubation with heparin at 38.5 °C. At both time points, an aliquot of spermatozoa was incubated with Indo-1 acetoxymethyl and FITC-peanut agglutinin, and then challenged with thapsigargin to induce the intracellular cascade involved in the capacitation process. The percentages of live spermatozoa with high intracellular levels of calcium and reacted

acrosomes were determined by flow cytometry. Analysis immediately after thawing before the heparin incubation and thapsigargin challenge showed a similar percentage of acrosome-reacted cells and cells with high intracellular calcium between bull A and bull C. Following thapsigargin stimulation, a similar percentage of spermatozoa from each bull acrosome reacted, but semen from bull A showed an increase in cells with high calcium compared with bull C (23.3 ± 6.3% vs 13.3 ± 2.5%, respectively; $P = 0.14$) (Table 81.3). After five hours of incubation with heparin at 38.5 °C and without thapsigargin challenge, spermatozoa from bull A had fewer (10.5 ± 2.3% vs 24.1 ± 0.9%; $P < 0.01$) acrosome-reacted cells compared with bull C, but a similar percentage of cells possessing elevated calcium (5.3 ± 0.5% vs 5.7 ± 0.1%, respectively; $P = 0.4$) (Table 81.3). After the thapsigargin challenge, bull A still had fewer acrosome-reacted cells compared with bull C (10.5 ± 2.0% vs 23.1 ± 0.5%; $P < 0.01$), but also had a proportionally smaller increase in the percentage of cells having high calcium. These observations suggest that bull A spermatozoa could not capacitate in heparin-containing medium, demonstrated by fewer acrosome-reacted cells and high intracellular calcium sperm. Altogether, this analysis suggests that this specific lot of semen from bull A was not mature enough to reach its full capacitation process and fertility potential. Saacke et al. [61] described some compensable and uncompensable seminal deficiencies related to subfertility. Compensable factors include functional or molecular traits reflected in this case study by the low percentage of cells having reacted acrosomes or elevated calcium. This case could benefit from increasing the number of spermatozoa per straw of bull A to compensate its subfertility.

The second case studied was an adult bull (bull B) that exhibited extremely low fertility in the field (FERTSOL <−2) based on several different semen lots released from an SPC after standard QC tests. We firstly performed the basic CASA/flow cytometry tests on five different batches and compared the results with an average bull (bull C). All the lots studied would have passed our QC cutoffs. Surprisingly, total and progressive motility percentages were not significantly different in bull B compared with bull C (Table 81.4). On the other hand, acrosomal reaction, cell death, and mitochondrial activity were significantly different in some lots analyzed compared with bull C (Table 81.4). As these results could not explain the very low fertility of bull B, we conducted a sperm chromatin structure assay analysis

Table 81.3 Comparison of acrosome integrity and calcium level of live spermatozoa from bulls A and C before and after a thapsigargin challenge, both post-thaw and after five hours' incubation with heparin.

	Immediately post-thaw				After 5 hours incubation with heparin			
	−Thaps		+Thaps		−Thaps		+Thaps	
Bull	AR	High Ca	AR	High Ca	AR	High Ca	AR	High Ca
A	1.8 ± 0.7	3.1 ± 1.2	16.3 ± 3.8	23.3 ± 6.3	10.5 ± 2.3[a]	5.3 ± 0.5	10.5 ± 2.0[a]	25.3 ± 2.3
C	1.5 ± 0.2	1.1 ± 0.2	17.8 ± 4.0	13.3 ± 2.5	24.1 ± 0.9[b]	5.7 ± 0.1	23.1 ± 0.5[b]	36.6 ± 8.8

Values are mean percentages from three replications ± SEM. Different letters (a, b) on the same column represent significant differences, $P < 0.01$, Student's paired *t*-test.
AR, Acrosome reacted; High Ca, elevated calcium level; Thaps, thapsigargin.

Table 81.4 Comparison of sperm functions based on CASA and flow cytometry analysis of bull B and C immediately after thawing.

Bull	Batch	Total motility (%)	Progressive motility (%)	Acrosome reacted (%)	Dead (%)	Mitochondrial activity (%)	DNA fragmentation index (%)
B	1	57.5 ± 4.5	32.5 ± 2.5	19.1 ± 0.2	43.5 ± 0.1*	55.3 ± 0.2*	7.05 ± 0.09**
B	2	44.5 ± 3.5	24.5 ± 3.5	19.0 ± 0.2	51.7 ± 1.2**	46.7 ± 1.1**	5.76 ± 0.12**
B	3	63.5 ± 1.5	34.5 ± 2.5	17.9 ± 0.1	39.8 ± 0.7	60.0 ± 0.6	4.24 ± 0.01**
B	4	56.0 ± 0.0	32.5 ± 0.5	16.1 ± 0.5	38.7 ± 1.3	60.3 ± 2.1	4.83 ± 0.11**
B	5	61.0 ± 5.0	34.5 ± 1.5	14.0 ± 0.3*	35.0 ± 0.3	64.6 ± 0.4	3.85 ± 0.03**
C	1	60.5 ± 0.5	29.5 ± 0.5	17.6 ± 1.0	38.6 ± 0.9	62.7 ± 0.2	1.61 ± 0.16

Values are mean percentages from two replications ± SEM. Significant differences from control are represented by *, $P < 0.05$ and **, $P < 0.001$, Dunnett's multiple comparison test.

(as discussed in the section "DNA Integrity") on the same batches and compared results with those obtained by bull C. The DNA fragmentation index percentage was approximately two to four times higher in bull B than in bull C ($P < 0.001$; Table 81.4). High DNA fragmentation index percentage has been correlated with subfertility, mostly in humans [53, 62]. Therefore the very low fertility observed for bull B could be explained by his high level of DNA denaturation as measured by the sperm chromatin structure assay. In opposition to the first case study, chromatin aberration is a non-compensable deficiency in subfertility [61]. Increasing the number of spermatozoa per straw in this case cannot compensate for the extremely low fertility of bull B observed in the field.

Summary

Multiparametric analyses of the semen produced by SPCs with CASA and flow cytometry demonstrate very high predictive potential for semen quality and fertility. As fertility has multiparametric aspects, research and development of new markers to identify high-fertility semen needs to be extensive. Incorporation of new markers in a multiparametric approach will lead to a better evaluation of semen quality and fertility. In addition, the implementation of these tools in SPCs will help to standardize the QC procedures by eliminating the subjective aspect of semen evaluation. This will standardize semen produced within a SPC and within multiple SPCs for AI companies that have semen produced in multiple laboratories. Another application of these tools in the AI industry is the characterization of semen with high genetic value. In the AI industry, most of the semen produced arises from a few high genetic value animals. These highly demanded semen samples should be very well characterized to optimize production in the number of straws produced and in the quality of the semen available for the market. Overall, the AI industry will benefit from the implementation of CASA and flow cytometry in SPCs.

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Superovulation in Cattle

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The authors gratefully acknowledge Drs. Reuben Mapletoft and Gabriel Bó, authors of this chapter which appeared in the first edition of this book, for their substantial contributions to this manuscript. We also thank Drs. Mapletoft and Bó for their numerous contributions to the bovine embryo transfer industry.

Introduction

The objective of superovulation in cattle is to maximize the number of transferable quality embryos (TQEs) possessing a high probability of producing pregnancies [1]. Wide ranges in superovulatory responses and embryo yield have been reported in reviews of commercial embryo transfer records. In 2048 beef donor embryo collections, a mean of 11.5 ova/embryos with 6.2 TQEs was recorded [2]. Variability in both the superovulatory response and embryo quality was substantial; 24% of collections did not yield viable embryos, 64% produced fewer than average numbers of TQEs, and 30% of donors yielded 70% of the embryos. Embryo recovery from 987 dairy cows yielded slightly fewer ova/embryos, but there was similar variability in response [3]. The high degree of unpredictability in superovulatory response creates problems that affect both the efficiency and profitability of embryo transfer programs [4].

Variability in ovarian response to superovulation has been related to differences in superstimulatory treatments (e.g. gonadotropin preparation), batch and total dose of gonadotropins, duration and timing of superovulatory treatments, and the concomitant use of non-superovulatory reproductive hormones. Additional factors influencing superovulatory response, which may be more important, are inherent to the animal and its environment. These factors may include season of the year as well as donor nutritional status, reproductive history, age, breed, prior superstimulation, and ovarian status at the time of treatment initiation. While considerable progress has been made in the understanding of bovine reproductive physiology in recent years, factors inherent to the donor animal that influence superovulatory response are not yet well understood.

Gonadotropins and Superovulation

Factors affecting superovulatory response include source, batch, and biological activity of the exogenous gonadotrophins [5]. Three different types of gonadotropin preparations have been used to induce superovulation in the cow: gonadotropins from extracts of porcine or other domestic animal pituitaries, equine chorionic gonadotropin (eCG; formerly called pregnant mare serum gonadotropin [PMSG]), and human menopausal gonadotropin [5, 6]. Human menopausal gonadotropin did not offer any advantages in cattle [7] and has not been used commercially; therefore, it will not be discussed further.

Pituitary extracts contain follicle stimulating hormone (FSH). The biological half-life of FSH in the cow is estimated to be five hours or less [8], so it must be injected twice-daily to successfully induce superovulation [9, 10]. The usual treatment regimen is twice-daily intramuscular treatments with FSH for four or five days, with a total dose of 28–50 mg (Armour) of a crude pituitary extract, or 260–400 mg NIH-FSH-PI of a partially purified pituitary extract (Folltropin®-V, Vetoquinol SA, Lure, France). At 48–72 hours after initiation of treatment, prostaglandin $F_2\alpha$ (PGF2α) is administered to induce luteolysis. Estrus occurs in 36–48 hours, with ovulations beginning 24–36 hours later [11, 12].

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Equine chorionic gonadotropin is a complex glycoprotein with both FSH and luteinizing hormone (LH) activity [13]. Its half-life in the cow is 40 hours, and it persists for up to 10 days in the bovine circulation; thus, it is normally injected intramuscularly once followed by a $PGF_2\alpha$ injection 48 hours later [14]. The long half-life of eCG causes prolonged ovarian stimulation, an increase in the number of unovulated follicles, abnormal endocrine profiles, and reduced embryo quality [15–18]. These problems have been largely overcome by the intravenous injection of antibodies to eCG at the time of the first insemination, 12–18 hours after the onset of estrus [14, 19]. Recommended doses of eCG range from 1500 to 3000 IU, with 2500 IU by intramuscular injection commonly chosen.

In one study, groups of cows were treated with either 2500 IU eCG or 50 mg (Armour) pituitary FSH [10]. Ovulation rate and the percentage of cows with more than three TQEs was slightly higher in cows treated with FSH than with eCG. Although these results were in agreement with one previous study [20], others have found no differences in superovulatory response between pituitary extracts containing FSH and eCG [21, 22]. Endocrine studies revealed that eCG-treated animals more frequently had abnormal profiles of LH and progesterone [15, 23], which were associated with reductions in both ovulation and fertilization rates [24], as compared with FSH-treated cows.

Although folliculogenesis in mammals requires both FSH and LH, there is considerable variability in FSH and LH content of crude gonadotropin preparations [5]. Radioreceptor assays and *in vitro* bioassays have revealed variability in both the FSH and LH activity of eCG, not only among pregnant mares but also within the same mare at different times during gestation [13]. The effects of the FSH/LH ratio of eCG on superovulatory responses have been examined, and there was a positive correlation between the ratio of FSH/LH activity and superovulatory response. Lower ratios of FSH/LH activity reduced ovulatory response in immature rats, and LH added to eCG reduced superovulatory response in cows [5, 13].

Purified pituitary extracts with low LH contamination seem to improve superovulatory response in cattle. Three groups of dairy cows were superstimulated with an equivalent amount of 450 μg pure porcine (p) FSH and varying amounts of LH and showed that the mean ovulation rate and the number of total and transferable embryos increased as the dose of LH decreased [25]. High levels of LH during superstimulation can cause premature activation of the oocyte [16] which often leads to a decreased fertilization rate. Several experiments with an LH-reduced pituitary extract [26] utilizing doses ranging from 100 to 900 mg of NIH-FSH-P1 revealed no evidence of detrimental effects of dose on embryo quality [6, 27]. On the other hand, doubling the dose of crude pituitary extracts containing both FSH and LH resulted in significantly reduced fertilization rates and percentages of TQEs [6]. Collectively, data support the hypothesis that the detrimental effect of high doses of pituitary gonadotropins on ova/embryo quality is due to an excess of LH.

Although it is generally believed that some LH is required for successful superovulation, endogenous LH levels may be adequate. One study [28] with recombinantly produced bovine bFSH induced high superovulatory responses without the addition of exogenous LH. These data suggest that LH is not needed in superstimulatory preparations and that embryo quality may be superior with pure FSH. The very high fertilization and TQE production rates in the absence of exogenous LH suggest that administration of LH in superstimulation protocols, at any dose, may be detrimental to embryo quality. Further, these results and more recent results with recombinant bFSH [29] indicate that future progress in bovine superovulation can be enhanced by the use of recombinantly derived gonadotropins.

The effect of LH on superovulatory response has also been demonstrated in an experiment involving Brahman (*Bos indicus*) crossbred heifers superstimulated with 400 mg NIH-FSH-P1 containing 100, 16, or 2% LH [30]. Although the more purified preparations in this experiment caused higher superovulatory responses, there were obvious seasonal effects; responses with the most purified and intermediate preparations were superior to the least purified preparation during summer months, but only the most purified preparation was highly efficacious during winter months. These results appear to contradict one report [31] that superovulatory response and embryo quality in Holstein heifers were not affected by LH levels in cool weather, but that during heat stress a more purified preparation yielded more corpora lutea and significantly more fertilized ova and TQEs. It becomes apparent that stress is the common factor; *Bos taurus* breeds likely find summer heat stressful, whereas *B. indicus* breeds likely find winter temperatures stressful. In either case, the more purified extracts resulted in greater superovulatory responses during conditions of environmental stress.

Follicular Wave Dynamics and Superstimulation

Superstimulatory response typically is greater if exogenous gonadotrophin treatment is initiated before selection of a dominant follicle. Recombinant bFSH given to heifers on day 1 of the cycle (ovulation = day 0), before the time of

selection of the dominant follicular of wave 1, resulted in more ovulations than when given on day 5 (after the time of dominant follicle selection) [32]. A subsequent study was conducted to determine if exogenous FSH given at the expected time of the endogenous wave-eliciting FSH surge had a positive effect on the superstimulatory response [33]. As the endogenous surge of FSH was expected to peak one day before wave emergence [34], superstimulatory treatments were initiated on the day before, the day of, or one or two days after follicular wave emergence. More follicles were recruited when treatments were initiated on the day of or on the day before follicular wave emergence, and more ovulations were detected when treatment was initiated on the day of wave emergence rather than the day before or one or two days after wave emergence. A subsequent study indicated that superovulation was induced with equal efficacy when treatments were initiated during the first or second follicular waves and that the superstimulatory response was optimized when treatment was initiated at the time of follicle wave emergence [35].

Superstimulation: The Traditional Approach

In the very early days of bovine embryo transfer, treatment with eCG was made to coincide with natural regression of the corpus luteum (i.e. approximately day 16 of the cycle) [12]. With the introduction of an exogenous $PGF_{2\alpha}$ commercial product in the 1970s, it became possible to initiate gonadotropin treatments at other times during the estrous cycle; most practitioners began treating donors with FSH during mid-cycle (i.e. 8–12 days after estrus) [36, 37]. Although this approach was initially based on anecdotal evidence, and then some experimental data [38], it is now known that this encompasses the time of emergence of the second follicular wave in cattle exhibiting two or three ovarian follicular waves per estrous cycle [39, 40].

Many practitioners prefer to use a descending FSH dose superovulation protocol (which mimics the naturally occurring decline in circulating FSH levels caused by inhibin near the time of ovulation) and treat donors with $PGF_{2\alpha}$ on the third day of the FSH treatment protocol. Others prefer to treat with $PGF_{2\alpha}$ on the fourth day of FSH, and many do not treat with FSH on the day after the administration of $PGF_{2\alpha}$. Although no differences have been found between four- and five-day FSH treatment protocols, recent experiments have shown that ovulation rate can be improved in some donors if FSH treatments are prolonged to six or seven days [41, 42]. Regardless, most superstimulation protocols have been successful in inducing superovulation under most circumstances [12]. Still others incorporate progestin into the protocol which ensures that donors do not come into estrus early, especially if it is not possible to confirm the presence of a corpus luteum before initiating FSH treatments. In all cases, inseminations are normally performed 12 and 24 hours after the onset of estrus [11, 37].

Although the initiation of superstimulatory treatments during mid-cycle has served the embryo transfer industry quite well over the years, conventional treatment protocols have two drawbacks: (i) the need for trained personnel dedicated to the detection of estrus, both before and after initiating treatments; and (ii) the necessity to have all donors in estrus at the same time in order to begin the superstimulatory treatments at the most appropriate time in groups of cows (i.e. mid-cycle). To obviate these problems, protocols that facilitate superstimulation subsequent to elective induction of follicular wave emergence have been developed.

Synchronization of Follicular Wave Emergence for Superovulation

Estradiol and Progesterone

The ability to electively induce follicular wave emergence permits initiation of superstimulation without regard to the stage of the estrous cycle and eliminates the need for estrus detection or waiting 8–12 days after observation of estrus in a prospective donor to initiate gonadotropin treatments [37]. In the 1990s the use of progestins and estradiol to induce synchronous emergence of a new follicular wave was reported [36], and its use in superstimulation protocols has been reviewed extensively [37, 43]. The estradiol treatment causes suppression of FSH release and follicle atresia. Once the estradiol has been metabolized, FSH surges and a new follicular wave emerges an average of four days after treatment [36, 44].

The most common protocol involves administration of 5 mg estradiol-17β or 2.5 mg estradiol benzoate, plus 100 or 50 mg progesterone by intramuscular injections at the time of insertion of an intravaginal progestin device (day 0) [37, 43]. Twice-daily intramuscular FSH treatments are then initiated on day 4. On day 6, $PGF_{2\alpha}$ is injected in the morning and evening, and the progestin device is removed in the evening. Estrus normally occurs on day 8 (approximately 48 hours after the first $PGF_{2\alpha}$ injection) and inseminations are performed 12 and 24 hours later.

Unfortunately, estradiol cannot be used in many countries around the world because of concerns about the effects of estrogenic substances in the human food supply [45]. This restriction leaves many embryo transfer

practitioners with a serious dilemma and created the need to develop treatments for initiation of a new follicular wave that do not involve the use of estradiol.

Follicle Ablation

An alternative to the use of estradiol in superstimulation protocols is to eliminate the suppressive effect of the dominant follicle by ultrasound-guided follicle aspiration of all follicles of 5 mm or greater diameter [46]. Follicle wave emergence occurs very consistently 24–36 hours later, and superstimulatory treatments are initiated at that time. This approach to the synchronization of follicle wave emergence for superstimulation is very efficacious and results in superovulatory responses that do not differ from the use of estradiol [46]. In addition, it is necessary to ablate only the two largest follicles to effectively synchronize follicle wave emergence [47]. The protocol involves the ablation of the two largest follicles at random stages of the estrous cycle and the insertion of a progestin device; FSH treatments are initiated 24–48 hours later and the remainder of the protocol is as described above. The disadvantage of ultrasound-guided follicle aspiration is that it requires ultrasound equipment and trained personnel, making it most practical when donors are held in an embryo production facility; it is difficult to routinely implement follicle ablation in the field.

Gonadotropin-Releasing Hormone

Another alternative to the use of estradiol for the synchronization of follicle wave emergence is to induce ovulation of the dominant follicle by treatment with gonadotropin-releasing hormone (GnRH) [48, 49], which is followed by wave emergence one to two days later [50]. However, emergence of the new follicular wave is synchronized only when treatment causes ovulation. When administered at random stages of the estrous cycle, GnRH results in fewer than 60% of animals ovulating [50, 51]. Not surprisingly, treatment with GnRH prior to initiating superstimulatory treatments at random stages of the estrous cycle resulted in lower superovulatory responses than treatments initiated after follicular ablation or estradiol treatment [52].

More recently, retrospective analysis of commercial embryo transfer data revealed no differences in the numbers of TQEs between donors superstimulated four days after treatment with estradiol and those superstimulated two days after treatment with GnRH (Randall Hinshaw, personal communication) [53, 54]. It is noteworthy that in each of these reports GnRH was administered two days after insertion of a progestin device. The improved responses may have been a consequence of progestin-induced development of a persistent dominant follicle that was more responsive to treatment with GnRH [55]. Obviously, controlled studies with the use of GnRH must be conducted to validate these promising results.

Improving the Ovulatory Response to GnRH

Most fixed-time artificial insemination (AI) protocols utilizing GnRH to synchronize follicle wave emergence employ a form of presynchronization to improve the ovulatory response to the first injection of GnRH [55, 56]. An alternative to that approach is to synchronize ovulation and then initiate FSH treatments at the time of emergence of the first follicular wave [33]. To avoid the need to detect estrus and ovulation in Nelore (*B. indicus*) donors, synchronous ovulation was induced with an estradiol-based protocol designed for fixed-time AI [57]. Gonadotropin treatments were then initiated at the expected time of ovulation (and emergence of the first follicular wave). Superovulatory response did not differ from a contemporary group superstimulated four days after treatment with estradiol. However, the number of TQEs was reduced in cows superstimulated during the first follicular wave unless accompanied by the use of a progestin device. A study with Holstein cows superstimulated during the first follicular wave also produced a greater number of viable embryos following the addition of a progestin device [58].

A series of experiments with the overall objective of developing a protocol for superstimulation following ovulation induced synchronously by the administration of GnRH was reported [59]. This approach was based on a previous study in which ovulatory response was increased by causing a persistent ovarian follicle to develop with the insertion of a progestin device 7–10 days before the administration of GnRH [55]. Ovulation and follicle wave emergence occurred one to two days after the administration of GnRH, indicating that this approach could be used in groups of randomly cycling donors.

The recommended superstimulation protocol is schematically presented in Figure 82.1. It consists of the administration of $PGF_{2\alpha}$ at the time of insertion of a progestin device. Seven days later (with the progestin device still in place) GnRH is administered to induce ovulation of the persistent ovarian follicle and subsequent follicle wave emergence; FSH treatments are initiated 36 hours after the administration of GnRH. Although this protocol was designed for four days of FSH treatments, a five-day superstimulation protocol can be accomplished by simply delaying the removal of the progestin device by one day. In this series of experiments, more than 95% of animals ovulated to the first GnRH administration,

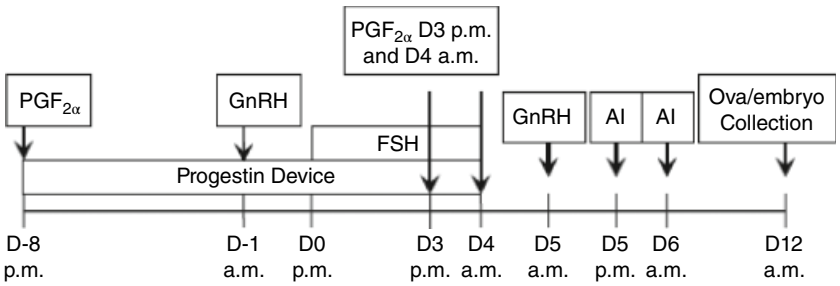


Figure 82.1 Treatment schedule for superovulation of donor cows during the first follicular wave after GnRH-induced ovulation. Donors receive a progestin device along with $PGF_{2\alpha}$ followed by GnRH seven days later. On day (D) 0 (36 hours after GnRH), superstimulation with FSH is initiated (twice-daily decreasing doses over four days). $PGF_{2\alpha}$ is administered with the last two FSH injections and the progestin device is removed with the last FSH injection. Ovulation is induced with GnRH 24 hours after progestin removal, donors are fixed-time inseminated (AI) 12 and 24 hours later, and ova/embryos are collected 7 days later. *Source:* From [59].

and superovulatory response and ova/ embryo numbers and quality were similar to that obtained when estradiol was used to synchronize follicular wave emergence [59].

Fixed-Time AI of Donors

A superstimulatory protocol specifically for *B. indicus* cattle (known as the P-36 protocol) has been developed [60]. In this protocol a progestin device inserted prior to the initiation of superstimulatory treatments is left in place for 36 hours after $PGF_{2\alpha}$ administration, and ovulation is induced by the administration of porcine LH (pLH) 12 hours after withdrawal of the progestin device (i.e. 48 hours after $PGF_{2\alpha}$ administration). Because ovulation occurs between 24 and 36 hours after pLH administration [61], fixed-time AI is scheduled 12 and 24 hours later, eliminating the need for detection of estrus.

In a series of experiments in which the timings of ovulations were monitored ultrasonically, a protocol for fixed-time AI in *B. taurus* donors without the need for estrus detection and without compromising results was developed [11]. Basically, the time of progestin device removal was delayed to prevent early ovulations and allow late-developing follicles to "catch up," followed by induction of ovulation with GnRH or pLH. In this protocol follicular wave emergence was synchronized with estradiol and a progestin device on day 0 and FSH treatments were initiated on day 4. On day 6, $PGF_{2\alpha}$ was administered in the morning and evening and the progestin device was removed on the morning of day 7 (24 hours after the first administration of $PGF_{2\alpha}$). On the morning of day 8 (24 hours after the removal of the progestin device), GnRH or pLH was administered and fixed-time AI was performed 12 and 24 hours later. Delaying the removal of the progestin device until the morning of day 7 resulted in a higher number of ova/embryos and fertilized ova than removal on the morning of day 6 [62]. From a practical perspective, fixed-time AI of donors eliminates the need for detection of estrus [63].

Studies in high-producing Holstein cows (*B. taurus*) in Brazil indicated that it is preferable to allow an additional 12 hours before removing the progestin device (i.e. evening day 7; P36), followed by GnRH or pLH 24 hours later (i.e. evening day 8) [64]. In *B. indicus* breeds, it was preferable to remove the progestin device on the evening of day 7 (P36), followed by GnRH 12 hours later (i.e. morning day 8) [65]. Although donors are typically inseminated twice, 12 and 24 hours after administration of pLH or GnRH [11], it is possible to use a single insemination with high-quality semen 16 hours after pLH [65].

Reducing the Need for Multiple Treatments with FSH

Because the half-life of pituitary FSH is short in the cow [8], traditional superstimulatory treatment protocols consist of twice-daily intramuscular injections over four or five days [9]. This approach requires frequent attention by farm personnel and increases the possibility of failures due to non-compliance with treatment protocol. In addition, twice-daily treatments may cause undue stress in donors, resulting in a decreased superovulatory response and/or altered preovulatory LH surge [66, 67]. Simplified protocols may reduce donor handling and improve response, particularly in less tractable animals.

A single subcutaneous dose of exogenous FSH can induce a superovulatory response equivalent to the traditional twice-daily treatment protocols in beef cows in high

Table 82.1 Mean (±SEM) ova/embryo production in beef donors treated with Folltropin-V given by twice-daily intramuscular injections over four days (control) or diluted in 2% hyaluronan and given by a single intramuscular injection.

Treatment	*N*	Total ova/embryos	Fertilized ova	Transferable embryos	Cows with "0" transferable embryos (%)
Control (twice daily)	146	12.1 ± 0.6	8.8 ± 0.5	6.7 ± 0.4	12(8.2%)
Single injection	146	11.2 ± 0.8	8.3 ± 0.6	6.4 ± 0.5	19(13.0%)

Source: From [59].
Means and percentages did not differ ($P > 0.10$) [59].

body condition (i.e. body condition score >3 on a scale of 5) [68], but not in Holstein cows which had less adipose tissue (lower body condition score). However, superovulatory responses were improved in Holstein cows when the total dose of exogenous FSH was split into two; 75% of the FSH dose was administered subcutaneously on the first day of treatment and the remaining 25% was administered 48 hours later (when $PGF_{2\alpha}$ is normally administered during superovulation) [69].

An alternative for inducing a consistent superovulatory response with a single injection of FSH is to combine the pituitary extract with agents that cause the hormone to be released slowly over several days. These agents can be polymers that are biodegradable and non-reactive in the tissues, facilitating use in animals [70]. In a series of experiments in which FSH diluted in a 2% hyaluronan solution was administered as a single intramuscular injection (to avoid the effects of body condition), a similar number of ova/embryos was produced as compared with the traditional twice-daily FSH protocol [71] (Table 82.1). However, 2% hyaluronan is viscous and difficult to mix with FSH, especially in the field. Although more dilute preparations of hyaluronan were less efficacious as a single administration than the 2% concentration, their use was improved by splitting them into two injections 48 hours apart, as was described previously with subcutaneous injections of FSH.

The split intramuscular treatment protocol consists of diluting the FSH lyophilized powder with 10 ml of a 1 or 0.5% hyaluronan solution and the administration of two-thirds of the total dosage of FSH on the first day, followed by administration of the remaining one-third 48 hours later, when $PGF_{2\alpha}$ is normally administered [72]. When compared with the twice-daily treatment protocol (controls) in 29 beef cows superstimulated three times in a cross-over design, the number of TQEs with the split-injection protocol (1.0% hyaluronan: 5.0 ± 0.9; 0.5% hyaluronan: 6.1 ± 1.3) did not differ from controls (4.0 ± 0.8). Data were interpreted to suggest that splitting the FSH dose (in either concentration of hyaluronan) into two intramuscular injections 48 hours apart results in a superovulatory response comparable to the traditional twice-daily intramuscular injection protocol in beef cattle. Furthermore, the less concentrated solutions of hyaluronan were not difficult to mix with FSH, even under field conditions. Data from a commercial embryo transfer company have confirmed these results in beef cattle in the USA [73].

Summary

Although the development of protocols that control follicular wave emergence and ovulation has not eliminated the variability in superovulatory response, these treatments have had a positive impact on the application of commercial on-farm embryo transfer by permitting the initiation of superovulatory treatments at a self-appointed time. These protocols offer the convenience of initiating superstimulatory treatments quickly without the necessity of estrus detection. In addition these protocols have facilitated fixed-time AI of donors, thereby avoiding the necessity of estrus detection during the superstimulatory protocol. However, estradiol, which has proven to be most useful in the field, has been withdrawn from many veterinary markets around the world, leaving only follicle ablation (which is difficult to utilize routinely in the field) as a reliable method to synchronize follicular wave emergence for superstimulation. Although the synchrony of follicular wave emergence following the administration of GnRH or pLH at random stages of the estrous cycle has been too variable for superstimulation, several recent reports suggest that this approach should be revisited. An alternative may be to initiate FSH treatments at the time of emergence of the first follicular wave following GnRH-induced ovulation of a progestin-induced persistent follicle. Finally, a single or split-single intramuscular injection of FSH in hyaluronan can be used to induce a satisfactory superovulatory response in beef donors, simplifying the entire protocol and reducing animal handling and the stress associated with the administration of superstimulatory treatments.

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Embryo Transfer and Embryo Collection

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A Brief History of Embryo Transfer

Embryo transfer began with rudimentary experimentation dating back centuries. In 1672, a young Dutch physician and anatomist named Regnier de Graaf observed the blastocyst of a rabbit without the aid of a microscope. Though he died only a few years later at the age of thirty-two, his contributions built the foundation for the future of embryo transfer [1]. Embryos from other domestic animals were observed nearly a century later, but it was the experiments performed by Walter Heape at the end of the nineteenth century that significantly influenced the field of reproductive biology and made development of embryo transfer technologies possible [2].

In April of 1890, the first live embryo transfer rabbits were born in his private lab at his home in Prestwich, Manchester. This novel achievement prompted other animal scientists to continue embryo transfer experiments in rabbits through the late 1920s and early 1930s, which, in turn, inspired the study of cultured embryos and their respective outcomes. It was during this time that using embryo transfer for advancements in agriculture gained the attention of the scientific community.

The first documented use of embryo transfer in farm species occurred at the Agricultural and Mechanical College of Texas in 1934, by Drs Warwick, Berry, and Horlacher using sheep, goats, and hybrids from the two species [2, 3]. Artificial insemination (AI), developed in the 1930s, was proving useful in improving genetic selection and would later become vital to embryo transfer. The success of AI led to interest in applying embryo transfer procedures to cattle embryos for the benefit of the animal agriculture industry. Support for more research grew throughout the 1940s, but World War II significantly slowed scientific progress.

Later in December 1950, the research efforts of Elwyn Willett, the Bureau of Dairy Industry of the United States Department of Agriculture, and the Department of Genetics at the University of Wisconsin produced the first embryo transfer calf [3]. The success came at a cost, however; Willett noted that it was not possible at the time to apply the practice of embryo transfer on a wide scale.

It was not until the 1970s that non-surgical embryo transfer became sufficiently developed to be used in practice and became possible to perform on the farm. In 1971, Dr. James Dula and Dr. Duane Kraemer produced the first commercial calf for Livestock Breeders International (LBI), a company they had established. The first calf to be born from a frozen embryo followed in 1973 [3–5]. Later, maturation of oocytes and *in vitro:* fertilization (IVF) were areas of progress, but much of the work done with livestock was outshined by the novel developments in human IVF and the birth of the first "test-tube" baby.

The growth in the decade to follow was due largely to advancements in embryo micromanipulation, embryo splitting, IVF, and molecular biology, all of which revolutionized the techniques performed in the industry. Commercial use of IVF grew in popularity and literature on the subject increased exponentially throughout the 1980s [6].

From the 1990s to the present, embryo transfer technology and research has continued to grow. Embryo transfer is an effective management tool, and those that delve into the field find a thrilling new frontier of research and innovation.

Applications for Embryo Transfer

Disease control and lower costs for animal imports and exports are significant advantages of utilizing this technology [7]. The principal advantage of embryo transfer,

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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however, is increased reproductive capacity of a genetically superior heifer or cow. The most conventional function of embryo transfer is to produce large numbers of offspring from valuable donors. Donor cows may be valued because they have excellent production records, an outstanding show ring record, are related to superior cows or bulls, have a popular trait such as the polled trait, or are in limited numbers. With shorter generation intervals, embryo transfer also provides the opportunity to test individual cows for possible genetic defects. This could prove tremendously useful in evaluating dams of AI bulls for undesirable recessive genes.

In addition, these techniques have a variety of applications in research. The fertilizing capacity of sperm cells is currently being studied using IVF techniques associated with embryo transfer [6]. With the growth of the genetic engineering field, there is expected to be an increase in its usage.

Overview of the Embryo Collection Procedure

Timing

Since embryos are most versatile at day 6–7, most practitioners plan collections 6.5–7 days after first observation of the donor's standing heat. When several donors are to be collected on the same day, there will usually be at least 12–24 hours variation in the onset of standing heat among them. Since embryos may hatch by day 7.5–8.0 and become less useable, it is usually wise to collect the donors first that exhibit standing heat the earliest. Also realize that collection on or before day 6 may result in embryos being too immature to endure the freezing process very well.

Collection Media

Many commercially prepared collection media are available today. Some use a ready-to-go medium from Vetoquinol called Vigro Complete Flush™, which contains a surfactant (polyvinyl alcohol) and antibiotics (gentamicin and kanamycin). Other practitioners use lactated Ringers which they have available in their veterinary clinics and simply add 0.1% bovine serum albumin as a surfactant. For short-term holding, this appears to work well and is much more economic.

If embryos are collected for export, some countries will not allow animal byproducts such as bovine serum albumin to be added due to the scare over bovine spongiform encephalopathy [BSE]. In these collections, use of a complete medium that uses polyvinyl alcohol as the surfactant is acceptable.

Donor Preparation

Elevation shifts the viscera posteriorly and tends to lift the uterus upward for ease of accessing it for manipulation. Although it sounds like a good idea to reduce feed intake prior to the technician's arrival in order to lessen feces, one of an embryo transfer practitioner's best friends is a full rumen. The full rumen not only elevates the uterus upward and caudally, but also prevents negative abdominal pressure caused by an empty rumen. Negative abdominal pressure causes air to be sucked around your arm and into the rectum and colon during the flush, making manipulation of the uterus very difficult. Ramps and feed are two great friends.

Once the donor is in the chute or stanchion, a lidocaine (3–5 ml) epidural is administered using an 18-gauge × 3.75-cm needle. Generally, no preparation is needed for the epidural. Little is accomplished by clipping or washing the hair unless one is willing to surgically prepare the tailhead. Immediately after the epidural, palpate each ovary and record an estimate as to the number of corpora lutea that are on each. Unovulated follicles are identified as well as the size of ovaries if they are unusual. This information is helpful once the results are in, whether good or bad.

Tranquilization is another friend, in some opinions, an enemy in others. According to experienced practitioners, giving 10 mg xylazine subcutaneously (SC) or intramuscularly (IM) just before the epidural works quite well. Although it causes a slight increase in uterine tone, it is usually not considered to be a problem. It is possible to collect cows without xylazine, but experienced practitioners like it because it takes the edge off excitable cows; they will stand still throughout the collection process.

Avoid using disinfectants around the collection materials. Simply wipe the perineal region after the tail is tied off to the side. In the absence of water, wipe the perineal area clean with a disposable towel. Once prepared, the cow is ready for collection.

Embryo Flushing Equipment (and Supplier)

Note that the items on this list are suggested, and due to the unpredictable nature of company acquisitions, some or all suppliers may differ.

- Catheter: 52 cm silicone, 16 French with 5-ml cuff (Vetoquinol)
- Stylet: 60 cm stainless steel (Vetoquinol)
- Y tubing: 150 cm plastic tubing, one end with syringe tip and the other attaches to EZ Way filter (PETS Inc.)
- Disposable three-way plastic valve (PETS Inc.)
- 50- or 60-ml airtight syringe (PETS Inc.)
- EZ Way Embryo Filter (PETS Inc.)

- Obstetric (OB) Lube (local farm supply store)
- Sterile lube (PETS Inc.)
- Cervical dilator (IMV Technologies)
- Medium: Vigro Complete (Vetoquinol)
- 10-ml syringe to inflate cuff (local farm supply store)

Sterilization

Most equipment is sterilized by the manufacturer using gamma radiation. Ethylene oxide was found to be embryotoxic years ago and is no longer used. If catheters and tubing are not autoclavable, simple washing with Alconox™ laboratory detergent and several rinses with distilled water is sufficient. Many practitioners discard all equipment after single use. Some practitioners reuse as much as feasible, depending on price.

Embryo Collection

The choice of catheter is affected by the type of flush (horn or body), age of the cow, and size of the cow. Today we have many choices available to add to the practitioner's ease and comfort during the collection. A 52-cm silicone 16 or 18 French catheter with a 5-ml balloon is a common choice for most flushes. The 5-ml balloon can inflate up to 20 ml if needed and it gives a rounder, more defined shape than the more elongated effect the 30-ml balloon may produce (Figure 83.1). However, a larger (20 or greater) catheter can be passed through the cervix, giving an opportunity for increased flow and decreasing the chance of a blockage due to mucus.

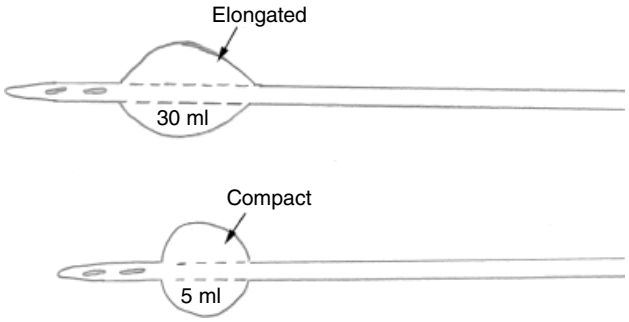


Figure 83.1 Shapes of Foley catheter balloon when inflated.

Catheter placement is the first critical step in non-surgical embryo collection. The placement of the catheter and collection procedures for both single horn and uterine body collection techniques will be discussed in further detail (Figure 83.2).

The following are important considerations. Is the donor a virgin heifer or cow? If virgin, how mature is her tract? Does the donor have a healthy reproductive tract or are there abnormalities? If abnormalities (e.g. adhesions, deformities, presence of infection, breed, age), what are they and how will they potentially affect the collection? What is the temperament of the donor? What are the facilities available for the flush? Thorough investigation prior to the procedure will benefit the practitioner and help determine whether to make adjustments.

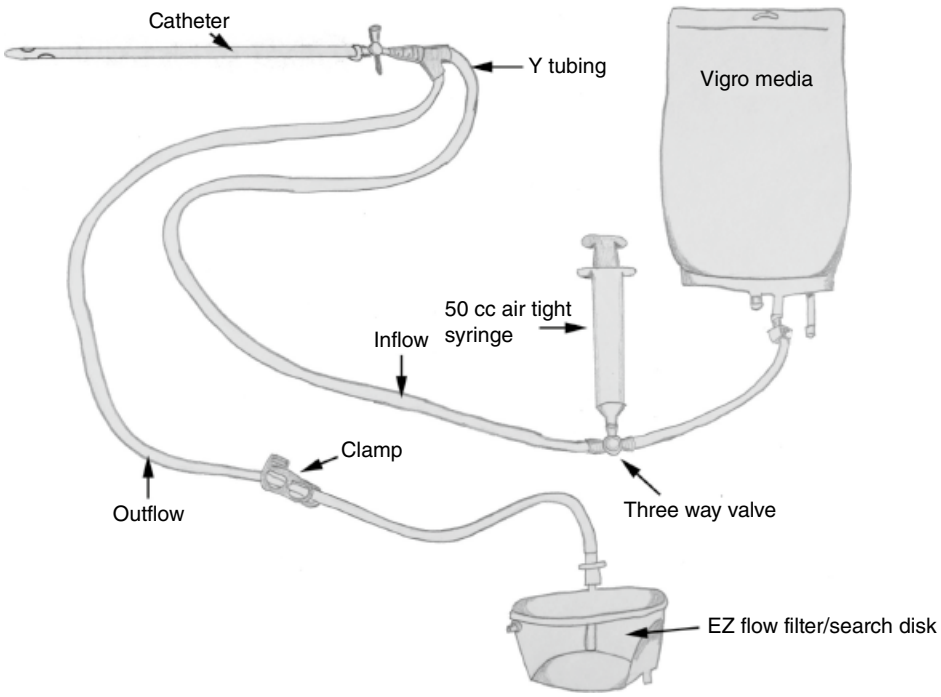


Figure 83.2 Schematic of flush equipment set-up.



Figure 83.3 Stylet protruding through catheter.

A metal stylet must be inserted into the catheter to make it rigid enough to be passed through the cervix. A small amount of KY jelly is applied to the tip of the stylet before entry into the catheter to aid in its withdrawal after passage. The stylet should be slightly longer than the catheter (60-cm stylet, 52-cm catheter) to allow the catheter to be stretched so the stylet will fit tightly inside it (Figure 83.3). This prevents the tip of the stylet from accidentally popping out of one of the fluid ports (holes) near the catheter tip during its passage through the cervix. Cervical damage and uterine hemorrhage are enemies. Passage of the catheter is similar to that in AI except the catheter is considerably larger. In addition, stretch must be kept on the catheter or finger pressure on the cervix may manipulate the stylet tip out of the lumen through the fluid port on the side of the catheter tip.

Eventually, every practitioner runs into a crooked cervix that cannot be passed with a catheter and stylet. An invaluable tool is a cervical dilator. The dilator can be passed through the most difficult cervix. Then, as the dilator is withdrawn by an assistant, one can quickly pass the catheter through before the cervix has time to return to its abnormal shape. Dilators are especially useful in Brahman and Brahman crossbreeds, which tend to get "S" curves and/or 90° bends in the cervix as they age (Figure 83.4).

Passing catheters through difficult or crooked cervices requires tenacity. Steady forward pressure must be kept on the stylet. Squeezing each cervical ring in front of the catheter often helps by "flattening" the ring, thereby making a "wide slit" out of the ring whose opening may be offset to one side or the other. The four cervical rings are usually aligned, with the opening of each ring being straight in front of the preceding one, but it is not uncommon to have one ring's opening in the center, the next at 9 o'clock, the next at 3 o'clock, and the last at 12 o'clock. Pressing holes into slits often aids passage. Dilators are diagnostic friends in discovering where each "hole" is located.

Body Flush

For the body flush, understanding the anatomy of the uterine body is necessary to prevent frustration (Figure 83.5). Depending on the age, breed, and size of the cow, the external bifurcation can be 5–15 cm from the cervical opening, causing practitioners to believe there is a lot of open space in the uterine body immediately anterior to the cervical os; however, this is not so. In reality, there is only 1.25–5 cm of open space (heifers usually 1.25–1.9 cm, while larger aged cows have 2.5–5 cm of space). Because the catheter has 2.5–3.75 cm of tip anterior to the cuff balloon, when the cuff is inflated the tip of the catheter protrudes into one of the horns. The balloon will occupy 1.25 cm with 2–3 ml of inflation but will occupy 2.5–3.75 cm if 6–10 ml inflation is required to make the balloon large enough so that it will not retract into the cervix. Catheter placement is easiest if you direct the catheter tip into the uterine horn opposite whichever arm you have in the rectum. For example, the practioner may use their left arm in the rectum, so it is easiest for them to place the tip of the catheter into the right horn.

Inflation of the cuff and seating the catheter's balloon is the *most important* part of a successful flush. Some veteran

(a) (b)

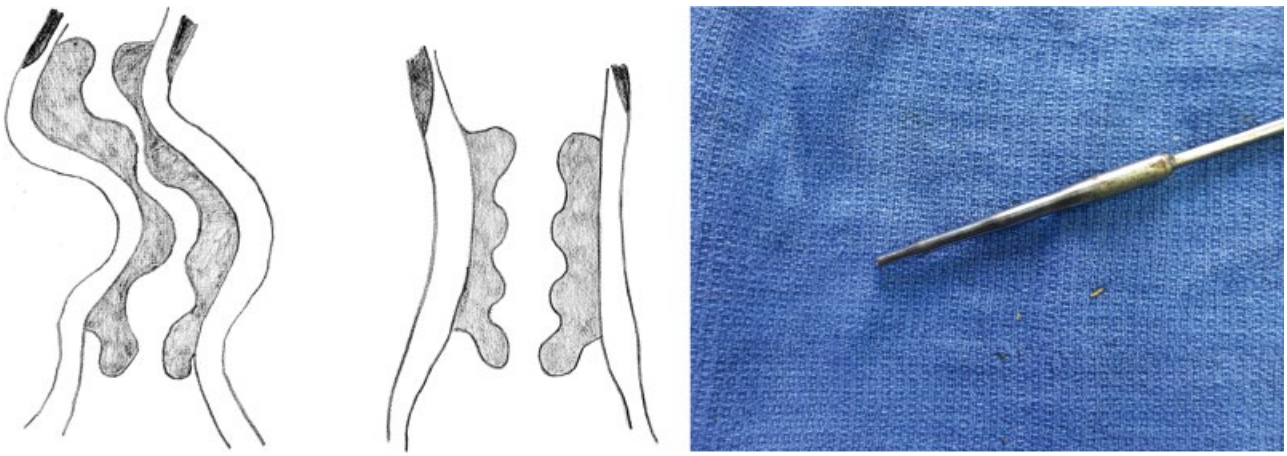
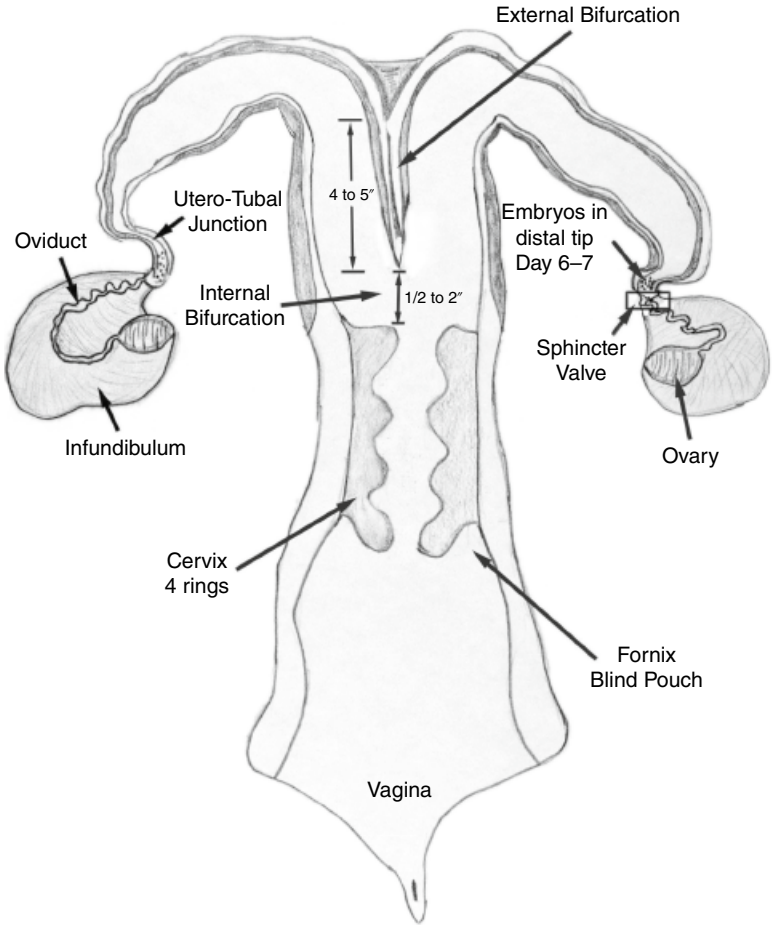


Figure 83.4 (a) Normal vs crooked cervix. (b) Cervical dilator tip.

Figure 83.5 Normal anatomy.



practitioners use flush media rather than air to inflate the cuff so they can see it dripping out of the valve if it leaks. The catheter should be gently introduced into the uterine horn 5 cm or so. Then, 1–2 ml of collection fluid should be injected through the valve into the cuff until you can manually feel the cuff expand. After the slightly expanded cuff is located with your fingertips, retract the stylet about 5 cm into the catheter and then gently retract the catheter until the cuff is in the uterine body just anterior to the cervical os. Once there, continue inflating the cuff. Gently tug the catheter caudally to see if sufficient inflation has been achieved to keep the cuff from retracting into the cervix – typically 2–3 ml is required in heifers and 4–10 ml in mature cows. Once satisfied that the inflation is correct, gently remove the stylet. (Note: Always put small amounts of KY jelly on the stylet tip when it is introduced into the catheter to prevent difficulty in withdrawing the stylet at this point.)

Overinflating and underinflating the cuff are both enemies. If underinflated, the cuff will pull back into the last cervical ring of the cervix as it relaxes. Even though the catheter tip will still protrude into the uterine body, the cervical pressure on the cuff will cause the catheter lumen to collapse and no fluid can be retrieved. If that happens, you must "pinch" behind the cuff in the cervix and squirt it back out into the uterine body where you can add more inflation. The only other option is to deflate the cuff, remove the catheter, reinsert the stylet, and start over. Usually it can be pinched forward without having to remove the catheter, which could result in loss of some embryos (Figure 83.6).

Overinflation results in only one horn being flushed unless some inflation is removed (Figure 83.7). Note that if a heifer or cow has only 1.9–2.5 cm or so in her uterine body, overinflation causes the cuff to engage both the cervical os and the leaf of the internal bifurcation, preventing fluid from entering both horns. Whenever this occurs, you will only be flushing one horn and unless you figure out what is not normal (i.e. "counterfeit") embryos will be left in the other horn.

Once the catheter is seated correctly, flush medium may be infused by gravity, syringe, or pump (Figure 83.8). It is recommended to use a 50-ml airtight syringe connected to a disposable three-way valve so it is possible to measure exactly how much fluid is being used. (Note: Prime the tubing to cut down

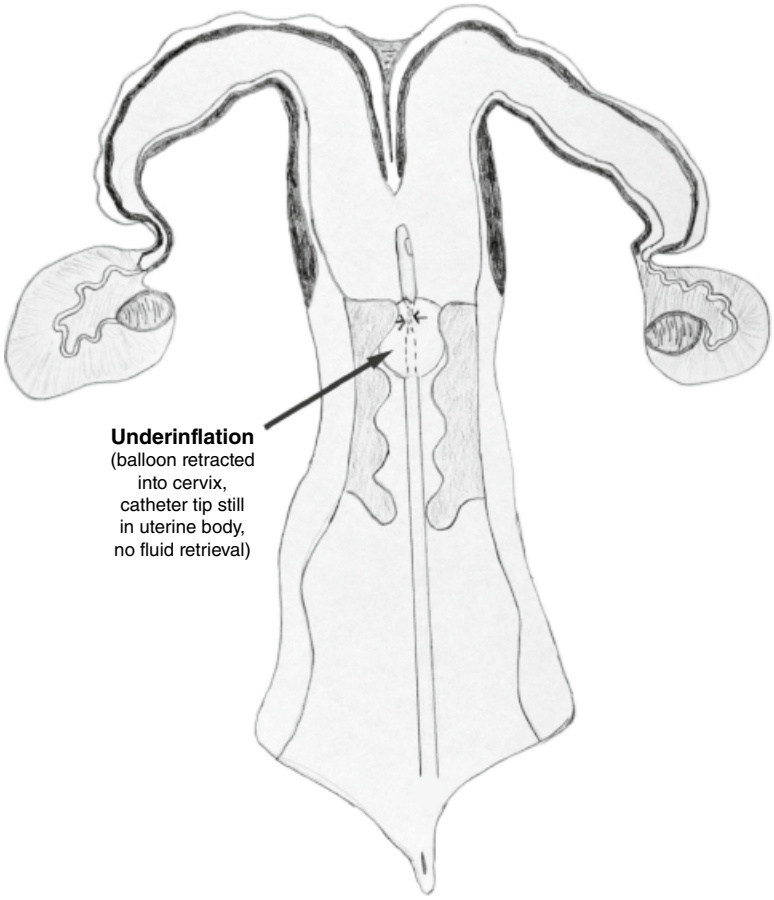


Figure 83.6 Underinflation.

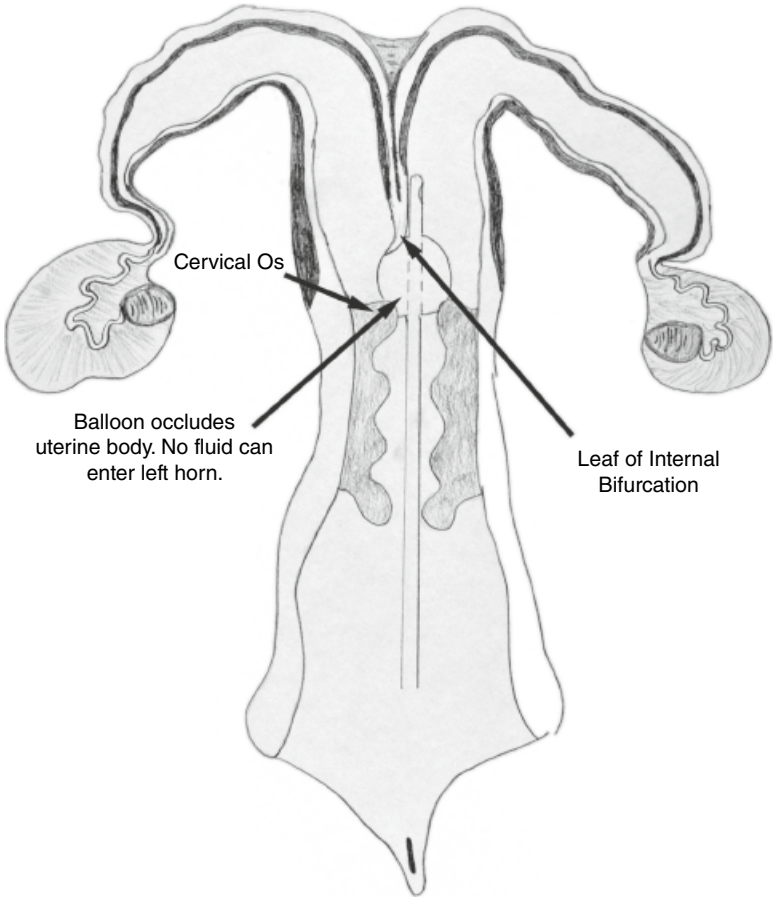


Figure 83.7 Overinflation and the occlusion of one horn.

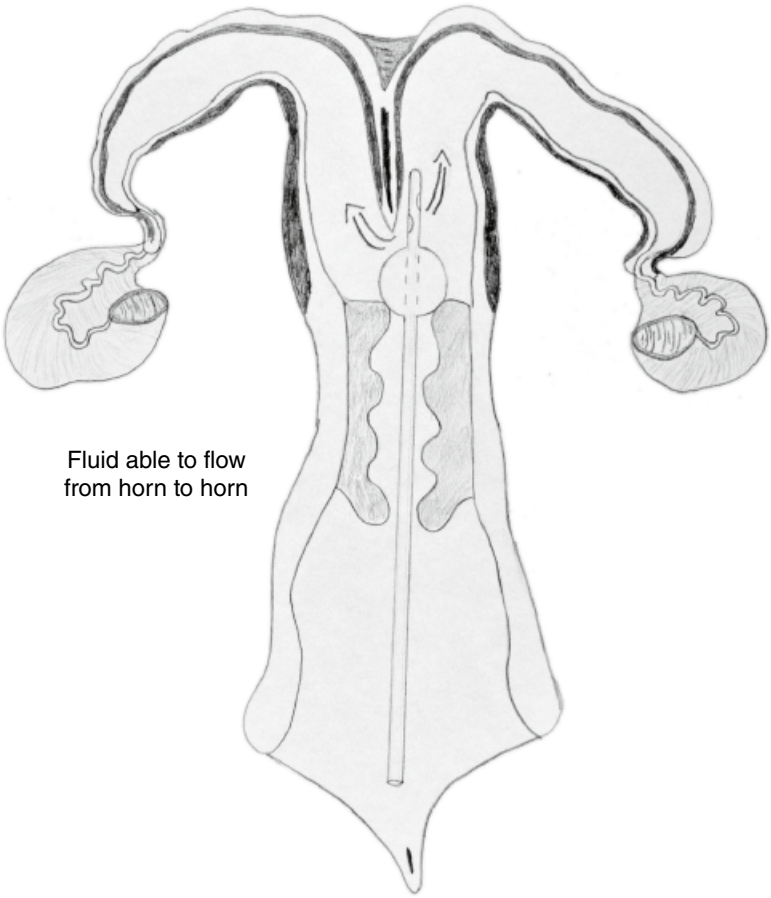


Figure 83.8 Correct catheter-balloon placement allows flush media to flow freely throughout both horns.

on air in the uterus. Figure 83.2 displays the contemporary set-up used for collections.) It is best to use a total of 400 ml of flush medium to collect both horns at the same time. Five infusions and retrievals are performed with the following amounts. Make sure to remove each infusion as completely as possible. Prior to the first infusion, the inflow side of the Y tubing should be filled (primed) with flush fluid to prevent air from being injected into the uterus, causing bubbles in the fluid.

- First infusion: 50 ml (25 ml into each horn), remove fluid.
- Second infusion: 50 ml (25 ml into each horn), remove fluid.
- Third infusion: 100 ml (50 ml into each horn), remove fluid.
- Fourth infusion: 150 ml (75 ml into each horn), remove fluid.
- Fifth infusion: 50 ml (25 ml into each horn), remove fluid.

Follow the flush with infusion of 25 mg Lutalyse through the catheter and into the uterus, deflate the cuff with a 10-ml syringe and gently remove. To assure that there are equal amounts in each horn, hold off the first horn just in front of the catheter tip. This forces the first half of each infusion to "dam up" and then run over into the other horn. Then allow the next half of that infusion to go straight ahead into that horn. You actually learn to "feel" the fluid running under your hand and into the opposite horn as it is injected via the syringe. If the fluid continues to "dam up" between your fingers and the cuff and does not run over into the opposite horn, the cuff inflation is too large and must be reduced sufficiently to allow fluid to flow between the cuff and the leaf of the internal bifurcation and into the opposite horn. Be sure the outflow side of the tubing is clamped closed before each infusion and then opened after you have massaged the fluid throughout the tract (Figure 83.9). After each infusion, the fluid is squeezed forward into the tip of each uterine horn and vigorously massaged to suspend the embryos. This massage forces media all the way to the uterotubal junction (UTJ) where the embryos are located.

Once the massage is completed, the clamp is removed from the outflow tubing and the fluid is retrieved. About half of the fluid usually runs out by gravity, while the other half must be reverse milked out. To reverse milk the fluid, generally it is recommended to clamp the horn near the

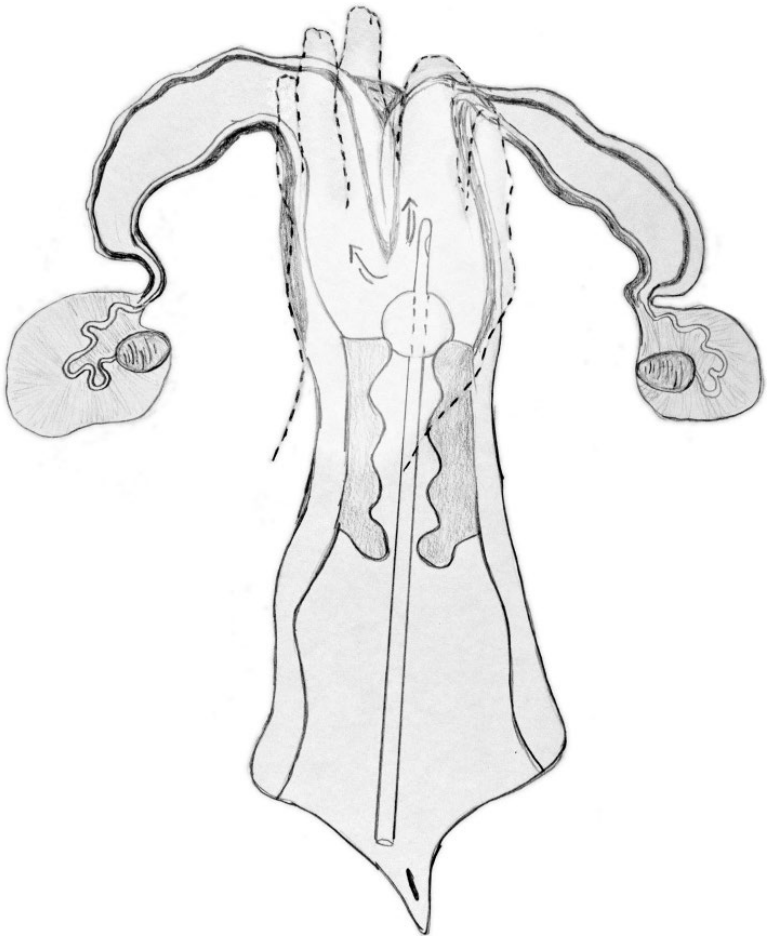


Figure 83.9 One horn can be held off to better fill the opposite horn.

uterine tip between the second and third fingers and use your thumb to pull fingers caudally with a motion similar to that of an inchworm. Alternate doing this between both horns and the fluid is easily moved back into the uterine body where it drains out the catheter. Sometimes you may gently tug on the catheter, which produces a pumping action in the body area that aids in fluid retrieval. Each person develops his or her own technique for fluid retrieval. Repeat this removal procedure after each infusion, trying to ensure the uterus is evacuated after each infusion.

Before embryo filters were available, technicians collected all the fluid and searched it all. It was very time-consuming to search 12 bowls or Petri plates for each flush. However, that method taught many practitioners a lot about their technique and efficiency (or lack of) in getting the embryos removed early in the flush. Beginners are encouraged to do the same today to build confidence in their technique.

To run this quality assurance assessment on yourself, use 12 plastic embryo transfer search bowls, each capped with a plastic lid that fits tightly (these lids can be acquired from a fast-food restaurant) (Figure 83.10). Drain infusion no. 1 into dishes 1 and 2, infusion no. 2 into dishes 3 and 4, infusion no. 3 into dishes 5 and 6, infusion no. 4 into dishes 7–10, and infusion no. 5 (the last) into dishes 11 and 12. By searching those dishes quickly, you can determine at what point in the flush you are retrieving the most embryos; 90% of the embryos will be found in the first six dishes if you are doing a good job. If you find embryos in dishes 11 or 12, you may have left embryos in the donor. By repeating the flush, you can determine if you have left any behind or not.

Note that using this technique the fluid will be too deep in each dish for you to easily see the embryos on the bottom. Since the embryos sink to the bottom quickly, you can use a 20-ml syringe to "skim" the top of the fluid until you have got the depth down to 0.6–1.25 cm so you can see the debris on the bottom of the dish easily. After you have searched every dish and recorded the number of embryos found per dish, pour all the flush fluid through a filter and find any embryos missed while searching the bowls.

To further illustrate the significance of this technique, take into account the following studies. In one study, 210 embryo recovery procedures were conducted using a double uterine flushing method, and the results were compared

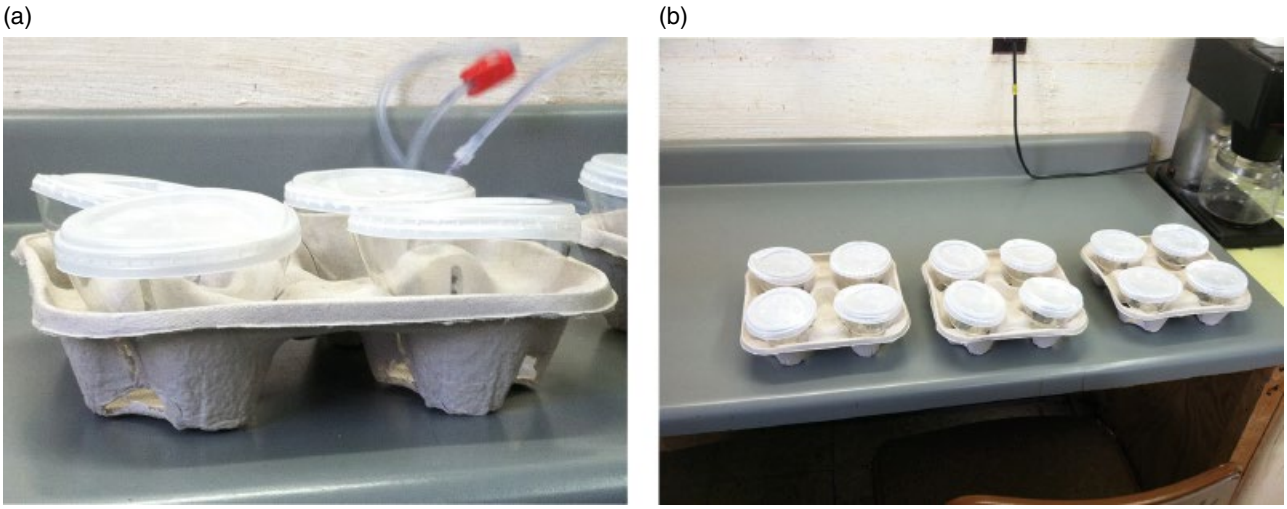


Figure 83.10 (a) and (b) Search bowls safely held in fast-food containers and covered with Solo™ cup lids.

with 432 conventional single-flushing treatments [8]. Double-flushing increased the average number of embryos recovered per procedure compared to single flushing procedures (6.7 ± 0.4 versus 4.6 ± 0.2, respectively). The results of this study highlight the underestimated value in reflushing donors.

In another study using 10 superovulated Holstein heifers, two different flushing techniques were used in a crossover design. At embryo recovery, one uterine horn was flushed with the deep method (about 7 cm caudal to the tip of the horn) and the other with the shallow (about 5 cm cranial to the beginning of the uterine bifurcation) [9]. The shallow flushing technique was superior to the deep technique for percentage of embryos or ova recovered per corpus luteum (63.9 ± 8.6 versus $37.4 \pm 6.5\%$).

One major lesson gleaned from this study was that the virgin uterus, having never been expanded by a pregnancy, often requires more dilation with fluid and massage to get fluid into that distal horn near the UTJ, which is where the embryos are on day 7. Although care must be taken not to overexpand the virgin uterus, causing hemorrhage or rupture, it must be expanded until full and tight and the uterine tips must be massaged to agitate fluid into that area.

The suggestion is for all novice practitioners to devise some way to evaluate their collection technique in order to develop confidence that all embryos are being efficiently collected. Collecting fluid into bottles and pouring the fluid through several filters is more costly but would be quicker than any method used in the era before filters.

Horn Flush

Note that the horn flush is considered an interrupted technique; you must remove the catheter and replace it, exposing it to the external environment. A body flush in contrast is considered a continuous closed system in which there is no contamination from the facility surroundings. This is an important distinction to consider when deciding whether to perform a horn flush.

Horn flushes require less flush medium, but their main value is in the single-egg flush or when oviductal blockage is suspected. For instance, if a cow had six corpora lutea on each ovary and only six embryos were recovered with a body flush, it is impossible to know where they came from. With a horn flush, if the fluid is searched separately from each horn, it is possible to diagnose oviductal blockage.

The horn flush is quick and safe in the hands of the experienced but has more potential pitfalls for the beginner. The key to a successful horn flush is to have a perfectly seated cuff in the horn each time. If the balloon is overinflated, the endometrium will tear, allowing flush medium and embryos to leak into the broad ligament, causing crepitation in the uterine wall and broad ligament that resembles the feel of blackleg-infected tissue. In addition, there will be a lot of hemorrhage to deal with. To salvage the flush, deflate the cuff, reinsert the stylet and go past the site of the tear, reinflate the cuff, and continue the flush.

If you underinflate the cuff, the catheter will slip caudally, eventually ending up at the cervix. In that event, you may choose to reinsert the stylet and reposition the catheter in the horn or just reduce cuff inflation enough to allow fluid to freely pass from horn to horn and switch to a body flush. When properly inflated one-half to two-thirds down the horn, the cuff will require 8–15 ml of inflation. Fingers should sense the cuff's expansion and inflation should stop when the cuff causes the horn to bulge slightly when adequately engaged; the cuff should feel slightly flattened and elongated rather than still round (Figure 83.11).

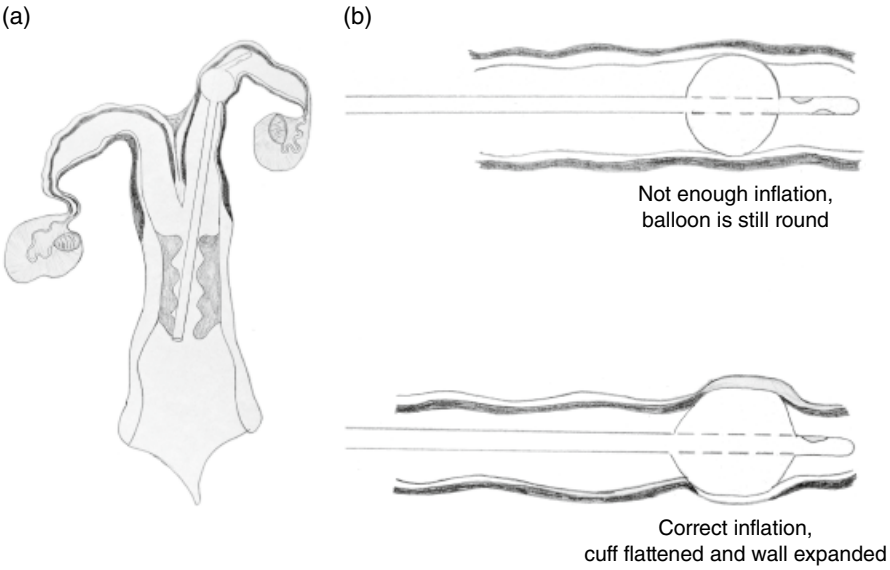


Figure 83.11 (a) and (b) Proper position and inflation for "horn" flush.

When properly positioned, the cuff should be as far as possible down the horn. Minimal amounts of fluid will be required for each flush. It is usually best to infuse medium five to six times into each horn tip with amounts that vary according to that necessary to achieve slight expansion of the horn. During each infusion, the uterine tip near the UTJ should be massaged before the fluid is retrieved. Most of the fluid will drain out by gravity flow, only slight manipulation being required to finish evacuation of all medium. Veteran practitioners usually begin with 15-ml infusions, increasing to 30–50 ml if the horn is large enough. When one horn is completed, the catheter is removed, the stylet reinserted, and the catheter then seated in the opposite horn. Experienced practitioners can rethread the stylet while the catheter is still in utero, but it is best not to try that at first.

The gravity flow technique of infusing medium is used by many practitioners, but some prefer to use syringes so they will have a known quantity infused each time to standardize the procedure. If gravity flow infusion is used, it is still necessary to massage the uterine tip after each infusion or embryos will be left behind. If collecting virgin heifers, vigorous massage of fluid into the UTJ area is a must. Some practitioners use injection pumps to infuse the fluid, but "simple" works well for others.

Filter and Search

Once the fluid is in the EZ Way filter, the filter is tilted so all the remaining flush fluid drains out through the filter mesh. Embryos are approximately 120 µm in diameter; therefore all the embryos should be on the 75-µm filter mesh on the side of the EZ Way. A 20-ml syringe filled with fresh flush medium with a 22-gauge × 2.5-cm needle attached can be used to spray the filter mesh off, allowing the wash to collect in the bottom of the EZ Way dish, which will then be used as the search dish. It is best to rinse the filter mesh off twice using 20 ml each time.

"Counterfeit Money"

In rare cases, abnormalities will be encountered and can be discouraging or even confusing for new practitioners. To think about these obstacles, consider the following metaphor. Experts train bank tellers to detect counterfeit money by first learning to identify what real money looks like, then they can spot any abnormality. The same holds true for embryo collection and transfer. Once you learn to collect normal cows efficiently and with confidence, then when trouble arises you will, hopefully, not panic but will smartly navigate through the trouble and salvage a successful but difficult collection. Some physical issues may be due to genetic abnormalities, AI technician damage, or self-inflicted trauma.

Genetic Abnormalities Encountered

Breed associations should be notified when these genetic abnormalities are found so that family lines that are carriers can be identified.

Unicorn Uterus

Strangely enough, some donors make it to the flush barn with only one uterine horn. This condition is called *uterus*

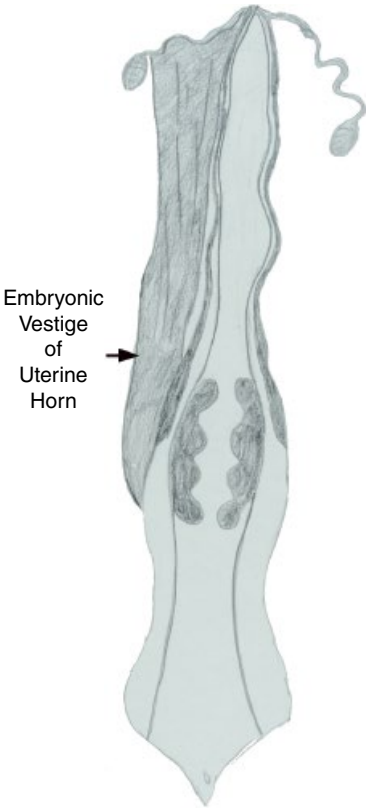


Figure 83.12 Uterus unicornis (segmental aplasia of the uterine horn).

unicornis. They are easy to flush because there is just one straight horn. Although many still have two ovaries, only half the number of embryos can be expected by corpus luteum count of the ovaries. The conclusion is that one ovary is connected to only a vestige of the missing horn while the other is connected to the existing horn (Figure 83.12). This condition is very likely an inherited defect.

Cervical Deformities

In time, all three of the cervical anomalies discussed below will be encountered. Cervical deformities can be a serious cause for frustration as it is vital to navigate this anatomical structure for a successful collection or transfer.

Y Cervix

The "Y" cervix has one opening in the vagina, and then somewhere midway through the cervix, the cervix bifurcates. Occasionally there is a common uterine body, but frequently each cervical opening goes to only one horn that does not communicate via a common uterine body. This means that, for both AI and embryo collection, each horn must be managed separately. Usually one of the cervical pathways is larger and unless the abnormality is diagnosed, all breedings will go to the same horn. Astute practitioners will notice the counterfeit cervix due to the lack of an internal uterine body. These are especially noticeable with recipients that have Y cervices. Non-professionals frequently artificially inseminate and even collect these abnormal uteri without knowing what was wrong (Figure 83.13).

Inverted Y Cervix

The inverted Y cervix has two openings in the vaginal vault, but both converge mid-cervix and only one opening is into the internal uterine body. These may be artificially inseminated and flushed, and if the practitioner is not astute they may not even be diagnosed as counterfeit but just classified as a difficult cervix to pass. These are not as critical for diagnosis since they share a common uterine body; therefore all embryos should be fertilized and collected regardless of which vaginal opening is used (Figure 83.14).

Double-Barrel Cervix

The double-barrel cervix has two openings in the vagina and two completely separate cervices, each going to its own horn. These are easier to diagnose by palpation. The cervix is noticeably wider than normal. With deep palpation, a groove can be felt which resembles the groove between the barrels of a double-barrel shotgun. Each "barrel" must be artificially inseminated separately and each horn collected separately since there is no common uterine body (Figure 83.15).

AI Technician Damage

Thickened Rectal Wall

When the donor for collection is palpated seven days after AI and a thickened edematous rectal wall is felt, it is a good sign the technician had difficulty with the AI. Occasionally, the rectum is so badly traumatized that it is impossible to safely collect ova from the cow. If collected, it is not unusual to collect non-fertile ova, indicating that the semen was never deposited in the uterus.

Brown Blood Clots

Finding brown blood clots in the first fluid retrieved usually indicates that the AI rod traumatized the uterine body resulting in mild to severe hemorrhage. The brown color indicates it is several days old, most likely from AI. This is another serious sign that embryos may not be fertile.

Infused Fluid Disappears, Not Retrieved

There are several reasons why fluid may not be retrieved during the embryo flushing procedure. First, there may be

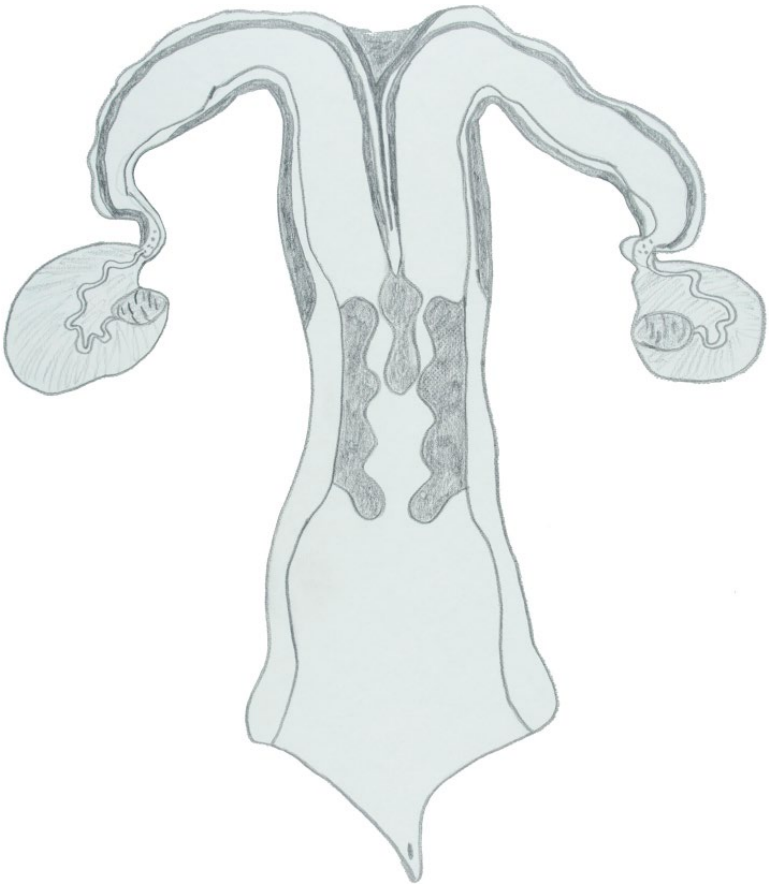


Figure 83.13 Y-shaped cervix (one external os, but a separate opening to each uterine horn).

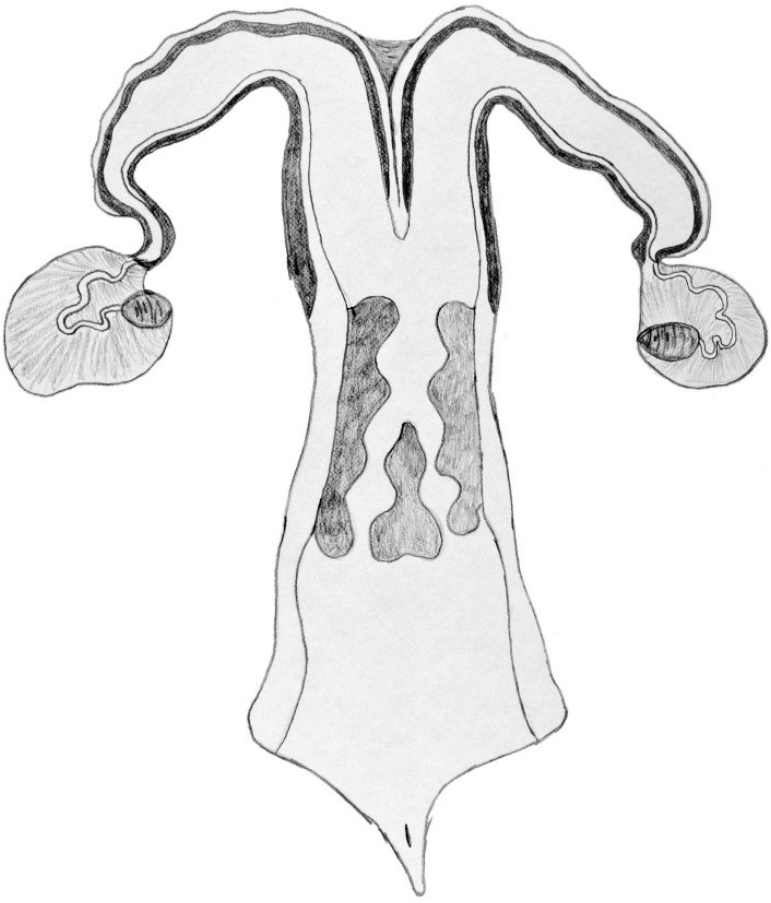


Figure 83.14 Inverted Y cervix (there are two external os, but only one internal os).

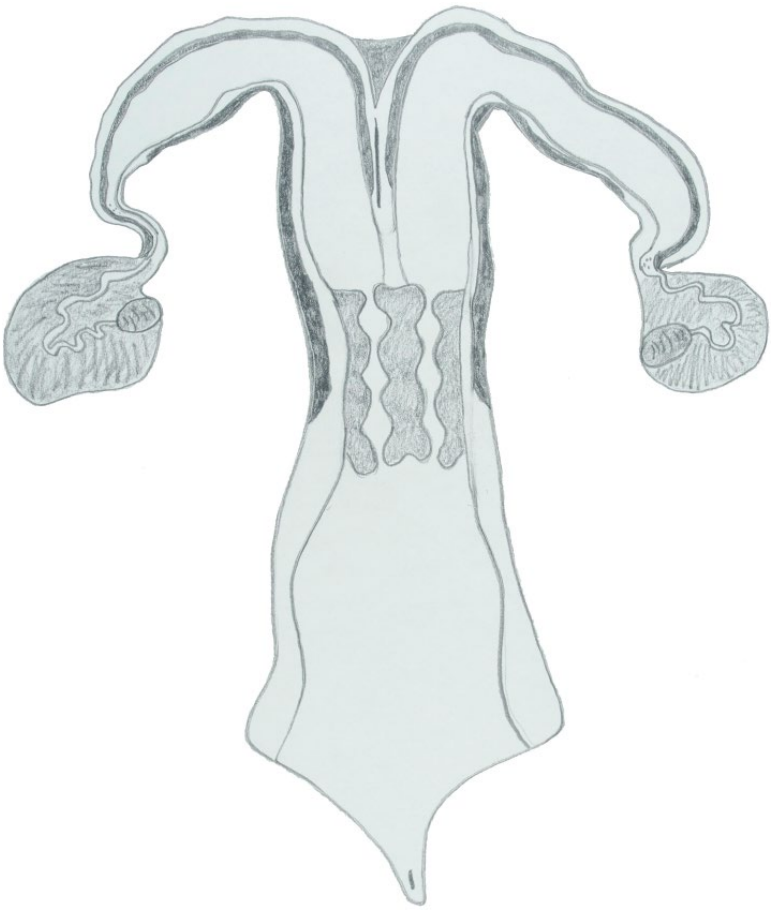


Figure 83.15 Double-barrel cervix.

a perforation from the AI rod that has not yet healed. Second, the wall may have been perforated. Previous trauma usually has brown blood clots whereas fresh trauma is always red. The flush can be salvaged in either case if the perforation can be located; just go past the hole, inflate the balloon, and flush horns separately.

The third cause is serious and almost always results in all embryos being lost. When many unovulated follicles exist that are full of active estrogen, the sphincter valve at the UTJ does not close properly on day 5 or so. When fluid is infused into the uterus on day 7, it simply flows through the UTJ, into the oviduct, and out into the abdomen, carrying all the embryos with it. This leaky valve is not common, but some veteran practitioners, including the author, have frequently encountered such cases. Some may not leak on early flushes of 25 ml per horn but will give way when the volume is increased to 50 or 75 ml per horn. This is reason to make the first two infusions of fluid a lesser amount and then increase volume on subsequent infusions. Consoling news is that these embryos are usually not fertilized anyway due to the increased estrogen level.

External Uterine Masses

A marble- to golfball-size mass found near the uterine body indicates probable trauma from the AI gun or harsh palpation resulting in hematoma. These will resolve in time, but if very large, they cause difficulty that day during the flush.

Pelvic Hematomas

Pelvic hematomas due to trauma from improper controlled internal drug release (CIDR) intravaginal placement may prevent you from accessing the uterus. These are generally unilateral, from either 3 o'clock or 9 o'clock, and protrude into the pelvic canal. Treat with systemic antibiotics for one week to prevent abscessation. If an abscess occurs, drain via vaginal puncture.

Embryo Transfer Practitioner Damage

Crepitation in the broad ligament indicates the endometrium has been torn, allowing flush medium to leak into it. The crepitation mimics the gaseous crepitation caused by blackleg in affected muscles. If encountered, stop the flush,

infuse the uterus with penicillin, and give systemic penicillin for five days. Embryos will be lost into the ligament. A frequent cause is slippage of the cuff backwards during horn flushes or overinflation of the cuff on either horn or body flush. It is an unforgettable feel. The palpable presence of crepitation is always a poor prognostic indicator for embryo recovery.

Hematomas can suddenly arise during the flush near the uterine body, especially in the bifurcation, or along the serosa of the horn if the practitioner handles the uterus too roughly. Always use "flat finger pressure" for palpation and avoid "fingertip pressure" as much as possible.

Other Problem Areas

Traumatic Adhesions

Traumatic adhesions have many etiologies. Cesarean section or dystocia that resulted in perforation can cause severe impingements, making uterine manipulation difficult to impossible. The presence of ovarian spider web-like adhesions is likely due to a previous ascending uterine infection or salpingitis. These are enemies and have a poor outlook. These spider-web adhesions often involve the ovarian bursa and interfere with ova pick-up during ovulation. The best long-term therapy is to try to get the cow pregnant by transplanting an embryo and hope the uterine expansion resolves enough adhesions to allow for sperm and ova transport through the oviduct. Alternatively, IVF could be attempted [6].

Sometimes a granular or sandpaper feeling to uterine serosa can be felt during catheter passage. This has an unknown origin but a distinctive feel when it is encountered. Trauma from CIDR "wings" may cause pelvic inflammation resulting in fibrin deposition on serosa. Most respond to repeated infusion of penicillin and systemic penicillin (100 ml daily for seven days).

Ovulation Tags

Ovulation tags, also called ovarian adhesions, are strands or "tags" of fibrin that can be secondary to ovulation. Ovulation tags generally do not impact reproduction and regress with time but can negatively impact reproductive performance if post-inflammatory adhesions are extensive [10]. In the case of severe ovulation tags, the resulting fibrosis may lead to inhibition of ovulation or oocyte transport.

Paraovarian Cysts

Small nodules around the ovarian bursa usually do not cause pathology unless they become very large. Most are less than 10 cm.

Preventing Unwanted or Multiple Pregnancies

Prostaglandin should be given to all donors post flush. Infuse 5 ml Lutalyse into the uterus at the end of the flush and then ask the owner to administer 5 ml (25 mg) IM twice a day on the third day following the flush. This quickly reduces ovarian size as well as aborting unwanted pregnancies. If cows are collected on 21- to 28-day intervals, it is very important to reduce ovary size quickly. However, infusing PGF2α into the uterus before the dishes are searched for embryos negates the ability to reflush the donor if needed. The IM injections twice on day 10 (3 days post flush) will be sufficient to prevent pregnancy if the owner can be trusted to remember to give the injections.

Helpful Hints

Ballottement Test

Even in normal passes of the catheter, occasionally a blockage occurs in the catheter. After the first fluid is infused and before any massage is done on the uterine tips, you can be sure the catheter lumen is patent with a simple test. With the fluid in the uterus before opening the exit clamp or palpating the tips, simply squeeze the bifurcation area gently two or three times. Movement of air bubbles from the catheter back into your clear tubing should be visible each time you squeeze, indicating patency. If no fluid or bubbles move caudally, there is a flow problem. If it cannot be corrected by massaging the catheter tip, adding or reducing cuff inflation, or moving the cuff around in the body, it is best to immediately remove the catheter and reintroduce a new one.

Washout Technique

When cervical passage of the catheter is difficult, quite often the catheter gets impacted with cervical mucus that enters through the side ports near the catheter tip. The mucus can easily be removed by a washout technique before any fluid infusion is allowed to go down the horns. With the catheter cuff inflated in the uterine body, wrap thumb and forefinger completely around both horns just anterior to the uterine body (Figure 83.16). Infuse 15–20 ml of medium through the catheter quickly and open the exit side of the tubing immediately, allowing that fluid to drain onto the floor. Repeat this two or three times until the fluid draining out the exit tubing is mucus-free. Remember, keep both horns pinched off so fluid only enters and exits from



Figure 83.16 Dr. Edwin Robertson's "washout" technique.

the uterine body. Disconnect the tubing from the catheter and completely wash out the tubing before reattaching to both catheter and EZ Way dish.

Cold Weather Outdoor Collections

To prevent chilling of medium, use two plastic stackable storage drawers from a department store. Each one should have a small electric heating pad added on the bottom of the drawer. The top drawer has the medium bag lying on the pad. In the bottom drawer we have the filter dish sitting on the edge of the pad at the front of the drawer where a hole has been made to allow the fluid to drain from the filter cup onto the floor.

To protect the tubing from the cow to the dish, use a 120-cm section of foam rubber water pipe insulation that has its side split open the entire length. Press the tubing inside the foam insulation section so the fluid can be kept relatively warm during the flush. In severely cold situations, an electric heat tape can be run through the piece of insulation as well.

Transferring the Embryo

Non-surgical embryo transfer is a great example of the old saying "A chain is only as strong as its weakest link." Many factors, some of which are beyond your control as the practitioner, will determine your success.

Selection for the Recipient Pool

It is frequently said that "the outside of a cow looks like the way her insides feel." Cows in poor general body condition with dull hair coats and kept in mud lots generally are not the best choice. So, what the embryo transfer practitioner looks for includes the following. (*Editor's note*: The selection and management of the recipient herd is covered in depth in Chapter 84. The following is an overview of areas that the authors feel are critical to the overall success of an embryo transfer program.)

Size

Dystocia can be a serious issue if proper precautions are not taken. Allow for the expected birthweight based on the breed of the embryo when adding cows to the pool.

Milk

Sound udders are ideal. While getting a live calf is important, it is also vital that the recipient be able to milk well and grow an exceptional calf. If transferring dairy embryos, this is not important since they will be bottle raised.

Prior History

Whenever possible, it is great to know that a recipient has calved regularly and has been an early breeder each year. Information is not available oftentimes though.

Lactation

Lactating cows in good body condition are usually great recipients. Usually, cows are usable by 45–50 days after calving *if* they are in good condition. Dry cows are excellent recipients if they have been held open, but if they are problem breeders or have already weaned a calf or been dried off, stay away from them.

Body Condition Score

The optimal body condition score (BCS) for fertility is 4–6 on a 1–9 scale. When ribs and spine are prominent on BCS 3 beef cattle, these are cows that are generally on the verge of going anestrus. They usually have smaller, firmer corpora lutea if they are cycling at all. These usually give disappointing results. Overly fat cows and heifers have gotten a bad reputation from the belief that "fat cows are infertile." This is not always the case. If the cow is fat because

she has been held open or been overfed, she may still have decent fertility. However, if a recipient has received two embryos or been exposed to several AI attempts without conceiving (three at most), she should be turned in with the bull or culled.

Mineral Deficiencies

Every area of the USA has certain mineral supplementation needs. You should know those in your region.

Disposition

Recipients must be handled often, so a good disposition is critical. This becomes especially important during the technical portions of the embryo transfer process.

Low Stress

Stressors need to be eliminated. Adequate shelter from cold in winter and heat in other months is necessary.

Biosecurity

Several diseases will wreck an embryo transfer program and are discussed in detail in previous chapters. Test all recipients and eliminate them from the herd if they are positive for one of the following diseases: bovine viral diarrhea virus (BVDV), *Neospora canis*, Johne's disease, or bovine leukosis.

Vaccination

All recipients should be vaccinated for BVDV, infectious bovine rhinotracheitis (IBR) virus, parainfluenza 3 virus, bovine respiratory syncytial virus, and leptospirosis. In my opinion, killed virus products are safer but not as effective. Modified live virus (MLV) vaccines with fetal protection for BVDV are best on open cattle. If MLV IBR vaccine is used, recipients will have lower fertility for 30 days due to the IBR virus colonizing the corpus luteum, resulting in decreased progesterone levels [11]. Be safe. A leptospirosis vaccine that includes *Leptospira borgpetersonii* (serovar Hardjo) must be added to the staple five-way leptospiral vaccine. This variant causes not only abortion, but also infertility and early embryonic death. It has become a major problem in most of the USA. Administer other vaccines as needed in your locale.

Recipient Synchronization

Estrus synchronization is discussed in Chapter 37. Use whatever means best fits the operation. Experience and data have shown that natural heats are no more fertile than induced heats. Many practitioners today use timed embryo transfer protocols, making heat detection unnecessary. This is a preferable method on farms with average management and poor heat detection. If a farm is well managed and has excellent personnel trained in heat detection, you may choose to use observed heats. In cases of very valuable embryos, observed heats are safer. Using Estrotect™ or another brand of heat-detection patches is highly recommended in this case.

Timed embryo transfer results in a slightly lower pregnancy percentage but produces more total pregnancies from a group of recipients. For example, if 100 recipients are in a group synchronized and heats are observed and recorded, approximately 85 will be seen in heat in the acceptable "window" or time frame for use. On average, 75 of those 85 will have good corpora lutea and will receive an embryo. If timed embryo transfer is used on 100 recipients, approximately 92 will have corpora lutea, so 92 embryos will be transferred. If the pregnancy rate is 60% for observed heats and 55% for timed embryo transfer, the total pregnancies produced will be 45 from the observed heat group and 50.6 from the timed heat group (plus the added advantage of no labor being spent on heat detection).

Acceptable Synchrony on Recipients

Generally, practitioners use heats on recipients that are 24–36 hours before the donor (8-day heats) to 24 hours after the donor (6-day heats). Of course, 7-day recipients are always best. Several considerations must be taken into account.

- Fresh embryos can tolerate greater asynchrony than frozen embryos. It is preferred to use day 7 (exact synchrony) to day 5.5 (36 hours after the donor) recipients for frozen embryos, while 5- to 8-day recipients are acceptable for fresh embryos.
- When more embryos are collected than recipients are available on a given day, the Grade 2 and Grade 3 embryos are usually transferred fresh while the Grade 1 embryos are frozen (see embryo evaluation in Chapter 85).
- Frozen embryos have undergone stress and therefore need closer synchrony. An 8-day recipient is rarely used, but there is no hesitation using a 5.5-day recipient for frozen embryos.
- IVF embryos are readily available today, both fresh and frozen. We use the same guidelines for synchrony on IVF as we do frozen embryos. However, IVF embryos usually are more advanced stages (expanded blastocysts stage 7 and hatched blastocysts stage 8). These embryos should be placed in recipients that are day 8.

Transfer Equipment (and Supplier)

Note that the items on this list are suggested products, and due to the unpredictable nature of company acquisitions, some or all suppliers may differ.

Frozen–thaw transfer:

- CITO™ Thaw unit (Nasco)
- Small Igloo (local store)

Frozen–thaw and direct transfer (DT):

- Scissors (local store)
- Sterile blue (metal tip) transfer sheaths ZT 133 (PETS Inc.)
- 2% Lidocaine HCl (Animal Health International Inc.)
- 18-gauge × 3.75-cm (1½-inch) needles (Animal Health International Inc.)
- Latex gloves (Animal Health International Inc.)
- Fingerless Maxi Sleeve (Animal Health International Inc.)
- ¼-cc Cassou guns – clean and straight (IMV Technologies or AgTech Inc.)
- Paper towels (local store)
- Chemise sanitary shield (IMV Technologies or PETS Inc.)
- 6-cc Monoject syringes (PETS Inc.)
- Sharpie and pens (office supply)
- Scotch Tape (local store)
- Obstetric (OB) lube (local farm supply store)
- Sterile lube (PETS Inc.)

For tracking recipients and record keeping:

- Z-tag blank (Animal Health International Inc.)
- Z-tag marking pen (Animal Health International Inc.)
- Ear tagger (Animal Health International Inc.)

Recipient Preparation

At this point, the first recipient finally goes into the chute. The same feeding principle applies to recipients as well as to donors: full rumens are your friends; empty rumens are enemies. Never withhold feed: a full abdomen makes transferring much easier.

When the recipient enters the chute, inject 3–5 ml of lidocaine as an epidural and quickly palpate for a good corpus luteum. Care must be taken once the epidural is given not to remove the arm from the rectum or a balloon-effect with air will occur. If the corpus luteum is good and the uterus is acceptable on palpation, load the embryo straw into a 52-cm IMV embryo transfer gun with blue sheath and immediately transfer the embryo. Always place an identification ear tag into each recipient, showing the mating and expected calving date.

New learners may wonder whether it is acceptable to move or transport recipients to and from the transfer site. If the weather conditions are very hot or very cold, short-distance hauls should have no bearing on pregnancy rate.

Site of Transfer

Embryos should be deposited into the uterine horn (ipsilateral to the corpus luteum) as deeply as possible without causing trauma (Figure 83.17). Prostaglandin is an enemy and is present in the endometrial cells. If the practitioner causes trauma as they pass the embryo transfer gun down

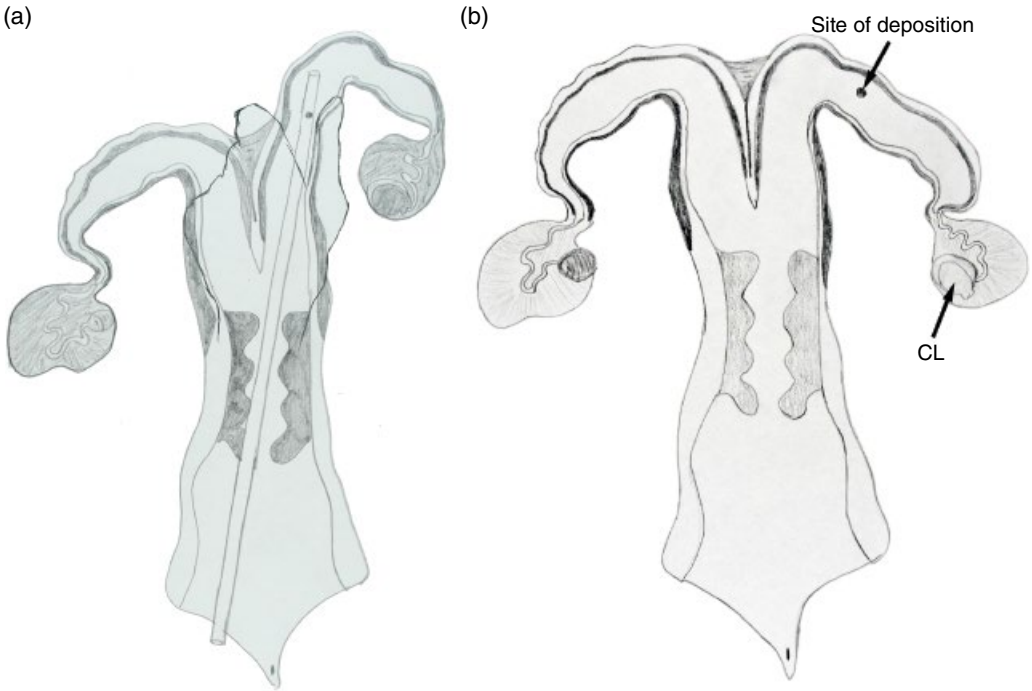


Figure 83.17 (a) and (b) Site of embryo deposition. CL, Corpus luteum.

the uterine horn, $PGF_{2\alpha}$ "leaks" from each damaged cell. Gentle flat-fingered pressure is necessary to smoothly guide the transfer gun without engaging or "bumping" the uterine wall. To do so requires the following conditions.

Restraint of Recipient

Some animals stand more quietly with their head not caught in a head gate, while others must be caught and squeezed. It is very frustrating to have the embryo near the drop zone and the recipient suddenly go berserk or be spooked by someone walking up near her head or yelling.

Footing

Solid footing is a major factor in an animal's willingness to stand still. Metal or slick concrete floors cause animals to panic and struggle. A good layer of bedding in the chute is helpful.

Climate

Overheated animals yield poor results. Every effort must be made to avoid the hot part of the day; alternatively, have fans and air moving. Plenty of water should be available. Minimal cattle movement should be done in the heat. Cold temperatures are generally not a problem unless extreme or if the conditions are also wet and muddy with no dry place to lie down. Recipient comfort is a friend.

Embryo Deposition: Gentle and Fast

Your technique inside the cow will evolve as you gain experience. Gentle and fast are the rules. Data have shown that time spent in passing through the cervix has little effect on pregnancy rates, but increased time spent going down the uterine horn usually affects success negatively. To achieve gentle and fast, the uterine manipulations experienced technicians have used will be described. The details are for being left-handed in the cow, so reverse the following procedure if you use your right hand inside. Here are the steps to follow after the recipient has received an epidural and been checked for a corpus luteum.

After the epidural and before placing your hand in the rectum, place the embryo transfer gun into the vagina and extend it to near the cervix. Once there, push the gun through the thin plastic wrapper that is over the blue sheath. Then place your hand into the rectum, locate the cervix, and stretch it forward, thereby removing any vaginal folds that may be obstructing the gun. Then pass the embryo transfer gun to the fourth cervical ring and hold it there. If the corpus luteum is on the right, before you pass the gun into the uterine body retract the cervix caudally and then reach forward and pull the right uterine tip upward and caudally; envision it as though a cobra snake is raised up to strike you in the face. Now, this horn will not stay there very long, so quickly pass the embryo transfer gun into the right horn and begin sliding the gun down the horn, having your four fingers on the medial side and ventral to the horn, your fingers being used to straighten the horn's wall as you continue forward. When you reach the curvature of the horn, use your thumb and forefinger to pull the horn forward and downward from its upright or sideways position, allowing you to get at least some distance around the curvature.

Each cow will be a different challenge. Some will allow passage almost to the uterine tip, while others must be deposited near the beginning of the curvature due to their size, fatness, etc. The key is to be atraumatic. Use flat finger pressure under the horn. Do not use fingertips to squeeze the horn and do not attempt to "thread" it onto the gun. Both of these movements will usually result in trauma.

For the left horn, start with your four fingers lateral and ventral to the horn with your thumb on top of the horn. Push the uterus slightly to the right in the pelvic cavity to enable you to enter the left horn atraumatically. Then continue down the horn while your fingers push the horn from left to right to allow for a straight path forward. When you reach the curvature, move the gun with your outside hand as far to the right as you can (which moves the gun tip to a left-sided direction internally). With the gun in the lumen at the curvature, wrap your fingers underneath the curvature to the medial side and cradle the curvature, bending it around straight as far as you can and slide the gun as far as possible without bumping or scraping the wall, and then deposit the embryo.

Always visualize yourself as sliding or laying the gun down into the horn rather than threading the horn onto the gun. When depressing the stylet to expel the embryo, envision yourself as laying a bead of toothpaste on your toothbrush. As you depress the stylet, retract the gun a slight distance in order to distance the tip from the embryo as it comes out and some like to rotate the gun 360° to "wipe" the embryo off the gun tip if, by chance, it was still adhered.

Frozen–Thawed Embryo Transfer

The transfer of frozen embryos is identical to that for fresh transfers except that frozen–thawed embryos should be transferred immediately on thawing whereas fresh embryos can be held several hours (six to eight) before transfer.

The preferred thawing process has been debated. The various options are a water thaw, air thaw, or a combination. In a water thaw, embryos from the liquid nitrogen tank (−193 °C) are plunged directly into a water bath at 35 °C. In an air thaw, embryos are held in the air until totally thawed. In combination thawing, embryos are removed from the liquid nitrogen tank, held in the air for five to six seconds, and then plunged into water at 35 °C. Putting embryos directly into water increases the possibility that the zona may crack, which may be undesirable for international work. Embryos may not be frozen for export if the zona is cracked, which could allow viruses or bacteria to gain access to the embryo. Allowing the straw to be held in the air for five to six seconds (or even a total air thaw) has been shown to prevent zona cracking. On a positive side, cracked zonas have been shown to be beneficial in the hatching process, since some embryos have been shown via microphotography to be unable to crack the zona (which enables or allows their growth), resulting in embryonic death. A combination of both methods may be the most efficient choice for some practitioners.

Most embryo transfer practitioners today use the IMV 52-cm embryo transfer gun with blue sheath. The sheath has a highly polished stainless-steel tip that causes much less endometrial trauma than the clear or green-plugged Cassou gun AI sheaths of the 1970s through 1990s. The side port on the IMV sheath (compared with the AI sheath's open end) helps prevent cervical debris from being injected right at the spot where the embryo is deposited.

Success rates for frozen–thawed embryos are very close to those for fresh embryos, usually within 5%, according to annual data published by the American Embryo Transfer Association (AETA).

Summary

Embryo transfer has an intriguing history, which exists in much more depth than most realize. It has led to many more developments in genetic selection and improvement, with additional advancements on the horizon. With the growing world population, novel developments in embryo transfer may play a more significant role and possibly necessitate a new, larger wave of experienced embryo transfer practitioners.

While extremely challenging, learning embryo collection and transfer, in addition to the many nuances of bovine reproductive physiology, can be equally rewarding. Individuals interested in becoming practitioners should strongly consider joining the AETA, the Canadian Embryo Transfer Association (CETA), or the Embryo Transfer Association in their respective country. Consider this quote from Dr. John Hammond:

> The economic success of the animal industry depends on the successful production and proper development of the next generation of our farm animals . . . experience gained on domestic animals will in the future enable man the more easily to control his development also. During the last 100 years great advances have been made in the physical sciences, as witness the steam-engine, motor-car, telephone and wireless. The experiments now proceeding in biological laboratories are beginning to show promise that during the next 100 years an equal advance will be made in the control of the animal.

Dedication

This chapter is dedicated to the lasting memory of the late Dr. Edwin G. Robertson who worked tirelessly to pave the way for future practitioners and students.

After graduation from veterinary school from Auburn University in 1974, he opened Harrogate Hospital for Animals. In 1977 he began practicing embryo transfer in the southeastern USA, eventually expanding to many distant foreign countries. He founded Harrogate Genetics International in 1989. From 1977 to 2014, he collected over 28 000 donors, transferred over 119 000 embryos, and froze over 86 000 embryos. He provided veterinary preceptorships for many veterinary students, becoming the first recipient of the "El Toro" Award (for excellence in food animal medicine) from Auburn University for his mentorship. He coordinated numerous teaching seminars for the American Association of Bovine Practitioners (AABP) from 1980 to 2013 and was named the AABP "Bovine Practitioner of the Year." Additionally, he received the Tennessee Veterinary Medical Association "Outstanding Practitioner Award."

Dr. Robertson was a member and served on the Board of Directors of the AETA. He was elected the 1989–1990 President of AETA. He served on the Board for the Society of Theriogenology from 1986 to 1992 and was President from 1991 to 1992.

Dr. Robertson dedicated his life to bettering his community and the world at large by improving the genetics of cattle and the quality of animal care and sharing his knowledge with the world. He was a true pioneer in assisted reproductive technologies and his work in embryo transfer was invaluable. His remarkable legacy will not be forgotten.

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Resources

American Embryo Transfer Association (AETA). www.aeta.org

Animal Health International Inc. www.animalhealthinternational.com

Canadian Embryo Transfer Association (CETA). www.ceta.org

IMV Technologies. www.imv-technologies.com

International Embryo Transfer Society (IETS). www.iets.org

Nasco. www.enasco.com

Professional Embryo Transfer Supply, Inc. (PETS Inc.) www.pets-inc.com

Vetquinol USA. www.vetoquinolusa.com

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Selection and Management of the Embryo Recipient Herd

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Introduction

The primary use of embryo transfer in cattle has been to amplify reproductive rates of valuable females. Ideally, embryo transfer can be used to enhance genetic improvement and to increase marketing opportunities with purebred cattle. Embryo transfer is especially useful with cattle because of their relatively low reproductive rate and long generation interval [1]. The success of embryo transfer depends on factors associated with the embryo, the recipient, and the embryo transfer technician, or an interaction among these factors. Once transferable embryos are collected from a donor cow, a decision is made as to which of the available recipients should receive embryos to achieve the greatest number of pregnancies [2]. Suitability of recipients is dependent on numerous management, nutritional, and estrous cycle control factors to ensure the presence of a functional corpus luteum (CL) at transfer. Although studies have focused on these factors, differences in techniques, sample sizes, and other elements have limited the applicability of the results of these studies. In many ways, management of the recipient is more critical to the success of an embryo transfer program than the donor, since the recipient is expected to conceive to the transferred embryo, maintain the pregnancy until full term, calve without assistance, and raise a calf of high genetic merit. This chapter focuses on recipient-related factors responsible for the success of an embryo transfer program.

Nutritional Management

Body Condition Score as an Indicator of Reproductive Efficiency

Insufficient intake of energy, protein, vitamins, and microminerals and macrominerals has been associated with suboptimal reproductive performance. Of these nutritional effects on reproduction, energy balance is probably the most important nutritional factor related to poor reproductive function in cattle. The metabolic use of available energy in ruminants for each physiological state is ranked, in order of importance, as follows: (i) basal metabolism; (ii) activity; (iii) growth; (iv) energy reserves; (v) pregnance maintenance; (vi) lactation; (vii) additional energy reserves; (viii) estrous cycles and initiation of pregnancy; and (ix) excess energy reserves [3]. Based on this list of metabolic priorities, reproductive function is compromised because available energy is directed toward meeting minimum energy reserves and milk production. Generally, beef cows do not experience a period of negative energy balance because they fail to produce the quantity of milk that dairy cows produce; however, beef cows need to be in sufficient body condition to resume estrous cycles after parturition and overcome anestrus, short estrous cycles, and uterine involution just to become pregnant every year.

Body condition score (BCS) is a reliable method for assessing the nutritional status of recipients. A visual BCS system developed for beef cattle uses a scale from 1 to 9, with 1

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

representing emaciated and 9 obese [4]. A linear relationship exists between body weight change and BCS, where an approximate 40-kg weight change is associated with each unit change in BCS. Managers of recipients should understand when cows can be maintained on a decreasing plane of nutrition, when they should be maintained on an increasing plane of nutrition, or when they can be kept on a maintenance diet. Understanding the production cycle of the cow and how to manipulate the diet will improve the ability of the recipients to conceive to the transferred embryo [5, 6].

Body condition score (BCS) at calving has been shown to be a more predictable indicator of the duration of postpartum anestrus than prepartum change in either weight or BCS [4, 7]. When cows were thin at calving or had a BCS of 4 or less, increased postpartum level of energy increased the percentage of females exhibiting estrus during the breeding season. BCS at parturition and breeding are the dominant factors influencing pregnancy success, although body weight changes during late gestation modulate this effect. However, altering poor body condition after parturition may reduce the negative impact on reproduction, but seldom overcomes or eliminates those negative effects. A study by Stevenson et al. [8] using blood samples at initiation of the breeding season to determine estrous cycling status demonstrated that only 47.2% of the cows were cycling at the onset of the breeding season. However, as BCS increased, the percentage of cows that were cycling also increased. It is important to note that when cows had a BCS of less than 4 at the beginning of the breeding season, only 33.9% had resumed their estrous cycles.

Prepartum Nutrition

The general belief is that cows maintained on an increasing plane of nutrition prior to parturition usually have a shorter interval to their first ovulation than cows on a decreasing plane of nutrition. Energy restriction during the prepartum period results in a low BCS at calving, prolonged postpartum anestrus, and a decrease in the percentage of cows exhibiting estrus during the breeding season [9]. Pregnancy rates and intervals from parturition to pregnancy are also affected by level of prepartum energy [9]. Conversely, when prepartum nutrient restriction was followed by increased postpartum nutrient intake, the negative effect of prepartum nutrient restriction was partially overcome; however, the effectiveness of elevated postpartum nutrient intake depended on the severity of prepartum nutrient restriction [7, 9]. The effect of BCS prior to calving also has implications for calf birth and weaning weights. When cows were fed to achieve a BCS of either 4 or 6 prior to calving, calf birth and weaning weights (with similar genetics) were greater for those cows at a BCS of 6 [10]. Despite the greater birth weights, there was no difference in calving difficulty, demonstrating the added advantage for recipients to wean calves with greater weaning weights. In addition, there tended to be an increased number of cows calving with a medium BCS that were cycling at the beginning of the breeding season and after a 60-day breeding season than cows in poor condition, resulting in a greater proportion of cycling cows at various stages of the breeding season [10].

Postpartum Nutrition

Numerous studies document that increasing nutritional levels following parturition increases conception and pregnancy rates in beef cows [4, 11]. Increasing the postpartum dietary energy density increased body weight and BCS, and decreased the interval to first estrus [7]. However, suckled beef cows in relatively poor body condition gaining in excess of 1 kg/day while consuming an 85% concentrate diet did not resume cyclic ovarian activity before 70 days postpartum [7]. Therefore, although an enhanced plane of nutrition after calving may partially overcome the negative effects of poor prepartum nutrition, the added stress and negative impact of suckling and lactation must be considered.

A major impact on postpartum fertility is the length of the breeding season. Having a restricted breeding season has many advantages, such as a more uniform and older calf crop, but most importantly a breeding season of 60 days or less increases the percentage of females cycling during the subsequent breeding season. If the breeding season is shortened, then all cows have a higher probability for pregnancy during the next breeding season. Strategic feeding to obtain ideal BCS can be achieved by understanding the production cycle of the cow. The period of greatest nutritional need occurs shortly after calving, when a cow is required to produce milk for a growing calf, regain weight lost shortly before and after parturition, and repair her reproductive tract to become pregnant within three months after calving. During this stage, a cow is usually consuming as much feed as she can, and attempting to adjust BCS at this time is often futile. Cows are usually grazing and tend to consume their full protein, vitamin, and mineral requirements; however, the grass is often lush with a high percentage of moisture, which can occasionally cause a deficiency in energy [12].

Estrous Cycle Control

Development of Estrus Synchronization Protocols

Factors such as nutrition, management, and inefficient detection of estrus affect the widespread use of embryo transfer in beef cattle operations. The most useful alternative for increasing the number of animals receiving embryos is to utilize protocols that allow for embryo transfer without the

need for estrus detection, usually called fixed-time embryo transfer (FTET) protocols. However, much of the research related to the systems currently used in embryo transfer programs was for fixed-time artificial insemination (TAI) rather than FTET. Transfer of embryos into estrus-synchronized cows has been most effective when embryos were transferred six to eight days after detected estrus or gonadotropin-releasing hormone (GnRH) injection [13]. Early estrus synchronization systems focused on altering the estrous cycle by inducing luteolysis with an injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) followed by estrus detection. Once systems involving a single $PGF_{2\alpha}$ treatment became successful, researchers focused on multiple injections of $PGF_{2\alpha}$ to further reduce days required for estrus detection [14, 15]. The next generation of estrus synchronization systems involved the use of exogenous progestins, such as an intravaginal controlled internal drug release (CIDR) insert or melengestrol acetate (MGA), which were used to delay the time of estrus following natural or induced luteolysis and extend the length of the estrous cycle [16, 17].

After the discovery of the wave-like pattern of follicular growth in cattle, scientists were able to embark on the third-generation systems of estrus synchronization [18]. Controlling follicular waves with a single injection of GnRH at random stages of the estrous cycle results in the release of a luteinizing hormone (LH) surge that causes synchronized ovulation or luteinization of dominant follicles [19–21]. Consequently, a new follicular wave is initiated in most (>60%) cows within one to three days after GnRH administration. Luteal tissue that forms after GnRH administration will undergo $PGF_{2\alpha}$-induced luteolysis six to seven days later [22]. A drawback of this method of estrus synchronization is that approximately 5–15% of cows are detected in estrus on, or before, the day of $PGF_{2\alpha}$ treatment, reducing the proportion of females that are detected in estrus during the synchronized period [23–25].

Advances in Protocols for Beef Cows

Preliminary studies identified significant improvements in fertility among cows that received MGA prior to the administration of $PGF_{2\alpha}$ compared with cows that received only $PGF_{2\alpha}$ [26]. When cows received a CIDR for seven days and an injection of $PGF_{2\alpha}$ the day before CIDR removal, estrus synchrony and pregnancy rates were improved [17]. In addition, when GnRH was given six or seven days prior to $PGF_{2\alpha}$, 70–83% of cows were in estrus within a four-day period [22].

The use of GnRH to control follicular wave emergence and ovulation and $PGF_{2\alpha}$ to induce luteolysis led to the development of the Ovsynch protocol for dairy cows [27]. Combining the second injection of GnRH with TAI (CO-Synch) proved to be more practical than estrus detection for beef producers because it had no negative effects on fertility [28]. However, a disadvantage of this protocol is that approximately 5–15% of suckled beef cows exhibit estrus prior to, or immediately after, the $PGF_{2\alpha}$ treatment [24]. Unless these cows are detected in estrus, they will fail to become pregnant to embryo transfer because of the lack of synchrony between the recipient estrous cycle and the age of the embryo. Therefore, the addition of a CIDR to a GnRH-based protocol can prevent the premature occurrence of estrus and result in enhanced synchrony between recipient estrous cycle and the age of the embryo. In the context of TAI, overall pregnancy rates were enhanced by the addition of a CIDR to a GnRH-based protocol (59 vs 48%, respectively). The CIDR delayed the onset of ovulation, resulting in more synchronous ovulation, and induced cyclicity in non-cycling cows [24]. Previously, the efficacy of these CIDR-based TAI protocols had not been evaluated concurrently with AI protocols requiring detection of estrus in suckled beef cows. Therefore, we implemented and coordinated a multistate, multilocation experiment to discern whether a GnRH-based with the addition of a CIDR protocol for TAI could yield pregnancy rates similar to protocols requiring detection of estrus [29]. Results demonstrated that the TAI protocols yielded pregnancy rates that were similar to the estrus detection protocol, even though 35% of the cows were in postpartum anestrus at the time of treatment.

A detailed version of current estrus synchronization and TAI protocols reviewed by the Beef Reproduction Task Force is available in Figure 84.1. Utilizing a similar protocol on recipients using FTET would be practical and effective in yielding high pregnancy rates in recipients.

Advances in Protocols for Beef Heifers

Early studies in beef heifers demonstrated that feeding MGA for 14 days followed by $PGF_{2\alpha}$ 17 days later was an effective method of estrous cycle control in heifers [16, 30]. However, when heifers were treated with $PGF_{2\alpha}$ 19 days after the 14-day MGA feeding period, there was no difference in fertility but estrus was more synchronous [31]. Following the success of this protocol, researchers began to include GnRH in estrus synchronization protocols for TAI. However, addition of GnRH to the above protocol failed to increase pregnancy rates following TAI in heifers [32]. Estrus synchronization using GnRH followed by $PGF_{2\alpha}$ successfully synchronized heifers, but the above MGA-$PGF_{2\alpha}$ protocol led to greater synchrony of estrus and therefore tended to be more effective [31].

Development of a TAI protocol in beefs heifers has not been as straightforward as in cows, especially considering that at the time of estrus synchronization the majority (>85%) of heifers have attained puberty [33]. The primary

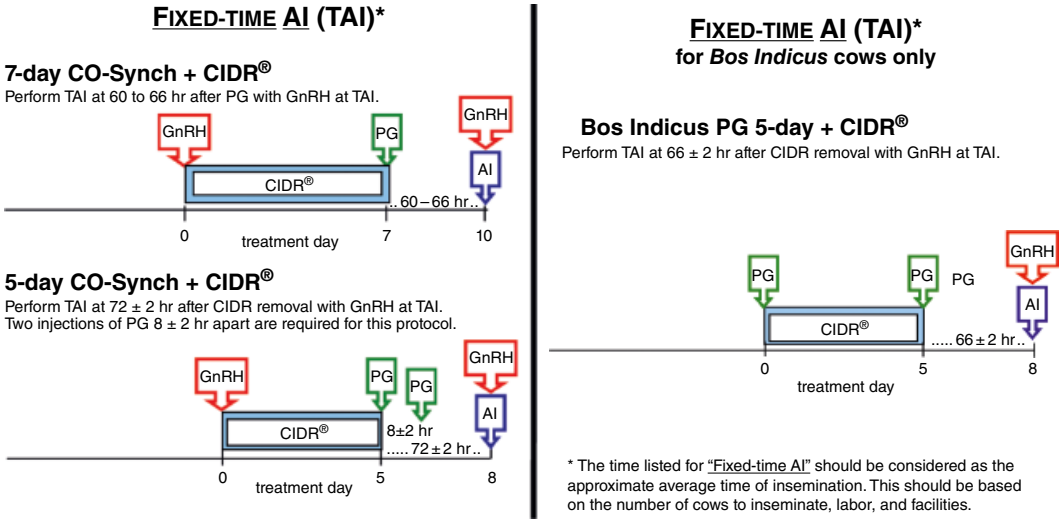


Figure 84.1 Current protocols for estrus synchronization and TAI of beef cows reviewed by the Beef Reproduction Task Force. Available at https://beefrepro.org.

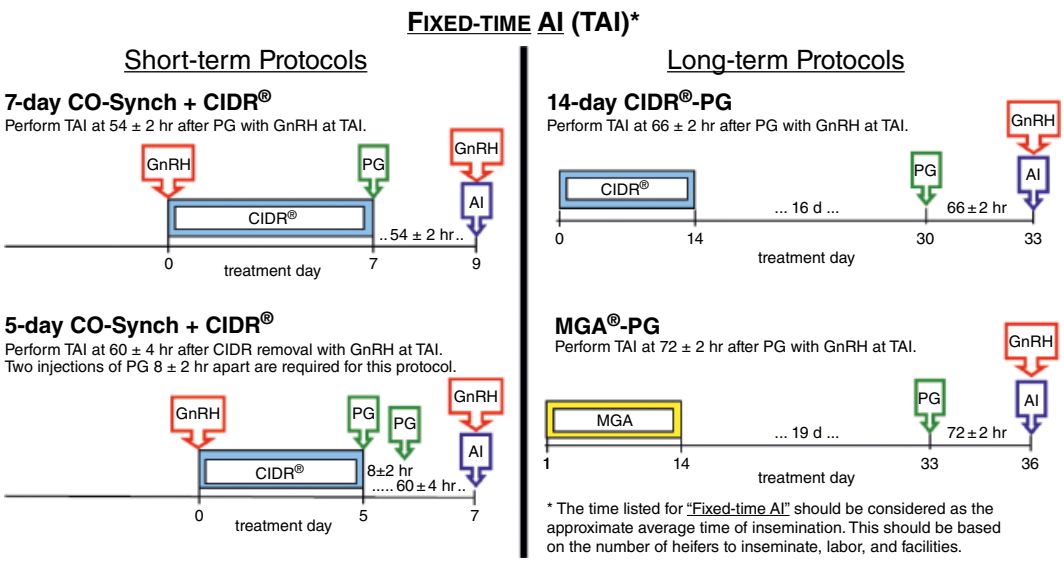


Figure 84.2 Current protocols for estrus synchronization and TAI of beef heifers reviewed by the Beef Reproduction Task Force. Available at https://beefrepro.org.

reason for failure of TAI in heifers appears to be the inability to synchronize follicular waves with GnRH. After an injection of GnRH at random stages of the estrous cycle, 75–90% of postpartum beef cows ovulated [34, 35], whereas only 48–60% of beef and dairy heifers ovulated in response to the same treatment [27, 36, 37]. We have found no difference in synchrony of estrus or pregnancy rate in CIDR-treated heifers whether or not GnRH is administered at CIDR insertion, suggesting that response to GnRH in heifers at CIDR insertion may be of limited value [33].

In a large multilocation study using GnRH, $PGF_{2\alpha}$, and CIDR, GnRH did not enhance pregnancy rates following estrus detection, but the addition of a CIDR to a GnRH-based TAI protocol yielded similar pregnancy rates to those utilizing estrus detection [33]. Nevertheless, a bewildering fact remains that the average pregnancy rate for these protocols ranged from 53 to 58%, whereas pregnancy rates in MGA (with $PGF_{2\alpha}$ administered 19 days after MGA removal) or long-term CIDR (with $PGF_{2\alpha}$ administered 16 days after CIDR removal) protocols followed by $PGF_{2\alpha}$ have been reported to range from 60 to 75% [23, 31, 33, 38]. A detailed version of current estrus synchronization and TAI protocols reviewed by the Beef Reproduction Task Force is shown in Figure 84.2. Utilizing a similar protocol on recipients using FTET would be practical and effective in yielding great pregnancy rates in heifer recipients.

Resynchronization of Estrus

Reinsemination of non-pregnant cows at the first eligible estrus can be facilitated by resynchronization of the estrous cycle [39], which would have wide application in intense embryo transfer programs. To most effectively condense the calving season, the second round of estrus synchronization needs to begin before the pregnancy status of the animals is known. Although resynchronization with a progestin increased synchronized return rates of non-pregnant females [40, 41], resynchronization with CIDR devices and estradiol cypionate or estradiol benzoate decreased subsequent conception rates to AI [40]. In contrast, further studies did not note a decrease in fertility when estrogens were utilized for resynchronization with a CIDR [42]. Furthermore, insertion of a CIDR for 13 days on the day of embryo transfer 7 days after estrus [43] or from 5 days after TAI until day 21 [29] was effective in resynchronizing estrus in non-pregnant cows, but insertion of a CIDR failed to enhance fertility compared to controls.

Recipient-Related Factors

Embryo Transfer Factors

The procedure of removing an embryo from its natural uterine environment, and in many cases freezing and thawing, increases the stress experienced by those embryos, resulting in a decreased survival rate following transfer. Results from our group (reference) show a decrease in pregnancy rate from 83% with fresh embryos ($n = 122$) to 69% with frozen–thawed embryos ($n = 326$) are similar to the 10–15% decrease in pregnancy rates reported previously [44, 45], which is similar to the difference in averages reported by the American Embryo Transfer Association (AETA) and the International Embryo Transfer Association (Savoy, IL). Results from two studies reveal that pregnancy rates among cows receiving a Grade 1 or Grade 2 fresh embryo [46, 47] were not different. Previous reports [2, 48–50] noted a decrease in pregnancy rate with each corresponding decrease in quality score.

Pregnancy rates have been shown to vary with the synchrony of the donor and recipient. Greater pregnancy rates were observed when recipients were in estrus coinciding with the donor or 12 hours before the donor. Pregnancy rates decreased when recipients exhibited estrus 12 hours after the donor [50]; however, other reports did not observe differences until 24 hours [45–47].

The variability of progesterone concentrations in recipients reflects a combination of different rates of CL development and the fluctuation of progesterone secretion during the early luteal phase. It has been suggested that the optimum circulating concentration of progesterone for establishing pregnancy ranges between 2.0 and 5.0 ng/ml [51]. However, a study has revealed that the minimum threshold progesterone concentration on the day of embryo transfer essential for the establishment and maintenance of pregnancy may be lower than previously reported, as there were no differences in pregnancy rates when progesterone concentrations were as decreased as 0.58 ng/ml or exceeded 16.0 ng/ml ($n = 448$) [47]. In another study, 8 of 177 pregnant recipients had concentrations of progesterone less than 0.5 ng/ml on days 10, 11, and 12 of the transfer cycle [52]. In addition, the diameter and volume of the CL differed among recipients that received embryos from 6.5 to 8.5 days after estrus [47], increasing as days after estrus increased. However, pregnancy rates did not differ among recipients receiving embryos 6.5–8.5 days after estrus.

A number of factors during the embryo transfer process have been reported to influence pregnancy success. Pregnancy rates have been inversely correlated with the time spent in the uterus during embryo transfer, where the least amount of time results in the greatest pregnancy rates [53, 54]. Furthermore, recipient movement while the embryo transfer gun is in the uterus increases the risk of damage to the endometrium, causing the release of $PGF_{2\alpha}$, which can hamper pregnancy success. When the prostaglandin synthesis inhibitor flunixin meglumine (FM) was administered at the time of embryo transfer, pregnancy rates were reportedly enhanced, but only when poorer-quality embryos were transferred [43, 55]. However, the United States Food and Drug Administration (FDA) has released a reminder that FM is only approved for intravenous use in cattle and extra-label drug use has resulted in increased residues found at slaughter. The site of embryo transfer has also been reported to influence pregnancy rates, where embryos that were transferred into the cranial part of the uterine horn tended to have greater pregnancy success when compared to those transferred to the middle and caudal third of the uterus [54]. Furthermore, transfers that are more difficult to perform tend to result in reduced pregnancy rates when compared to easier transfers [54].

General Recipient Considerations

Selection and identification of high-quality recipients is not simple. Many prefer the use of virgin heifers, whereas others choose cows with a known history of high fertility. When heifers are to be used as recipients, the selection criteria should be the same as for high-quality replacement heifers. Heifers need to be cycling, which can be assessed indirectly by using reproductive tract scores that are assigned based on the diameter and tone of the uterine

horns, as well as ovarian morphology (scale of 1 = reproductively immature to 5 = sexually mature) [56]. In addition, heifers need to be on an increased plane of nutrition, have an adequately sized and normally shaped pelvic canal, and have no history of receiving growth implants.

Lactating recipients have an advantage of a known reproductive history. Since the health of the calf is dependent on the recipient, records should be kept of calf health and weaning performance. Recipients that carry an embryo transfer calf to term but do not raise a normal calf to weaning should be reevaluated as a recipient prospect. Similarly, open cows with an unknown reproductive history need to be carefully examined prior to being included in a recipient herd or program [57]. The reproductive tract needs to be thoroughly examined via rectal palpation or transrectal ultrasonography for pregnancy or uterine anomalies such as fluid or fetal remnants, or evidence of metritis or endometritis, and the ovaries examined for normal follicular or luteal structures. In addition, recipients should have good teeth and eyes, a good udder, be less than eight years of age, and be structurally sound. Greatest fertility occurs in herds where handling facilities are designed to ensure that cattle are handled with minimum stress.

It is wise to keep the new arrivals separate from the breeding herd until sufficient time has elapsed for diagnostic screening tests to return and any incubating disease to become apparent. Many purebred producers and embryo transfer companies take blood samples to test for exposure to bovine leukosis virus (BLV), *Mycobacterium paratuberculosis* (Johne's disease), bovine viral diarrhea virus (BVDV), anaplasmosis, and *Neospora caninum*. Brucellosis testing or vaccination is no longer required in many areas, but it is prudent to test cattle from areas where the disease is present in wildlife. Many of these pathogens have been associated with decreased fertility by preventing fertilization or by causing embryonic death, fetal loss, or ovarian dysfunction [58]. A vaccination program to control livestock diseases is a common and proven practice. Conventional recommendations suggest that modified-live virus vaccines be given at least 30 days prior to breeding. However, recent research has shown that in previously vaccinated cattle, there are no detrimental effects of vaccinating cattle at the time of $PGF_{2\alpha}$ treatment or initial GnRH treatment [58]. Cattle with an unknown or questionable history of vaccination should receive primary and booster vaccinations at least 30 days prior to breeding [59].

Management after Confirmation of Pregnancy

Once the recipient is confirmed pregnant, she needs to be managed to remain pregnant. There are several environmental factors that cause abortion depending on the area of the country where the cow is maintained. Some of these include *N. caninum*, locoweed, ponderosa pine needles, fescue toxicity, nitrates, and mycotoxins and/or moldy feed. Handling stress has been shown to cause heifers to abort, but no reliable data have focused on the ideal interval after embryo transfer to transport recipients. However, pregnancy rates were lower in females that were transported between 8 and 33 days after AI as compared with those transported within the first 4 days [60]. Therefore it appears to be more desirable to transport recipients prior to embryo hatching than after hatching. Infectious agents (e.g. BVDV, infectious bovine rhinotracheitis, and, rarely, bluetongue virus) are also a threat to the developing fetus. Often it is not possible to diagnose the cause of abortions, but diagnostic success can be increased if the proper samples and history are submitted [61].

Biosecurity

To protect the herd from the introduction of infectious diseases that could cause abortion or reduce the value of the calves, a well-designed biosecurity program needs to be in place. This includes a test and quarantine program for all new cattle introduced into the herd. A commonsense program of single-use needles and rectal sleeves, disinfection of equipment between cows (drench applicators, tattoo pliers, and other instruments), external parasite control, the use of clean coveralls for employees and visitors as well as footbaths or shoe coverings, and not allowing equipment or clothing to be taken from one farm to another without first being thoroughly washed and disinfected can minimize risk of introducing pathogens onto a facility.

Pregnancy Diagnosis

Establishing a pregnancy diagnosis program allows for the detection of cows that are not pregnant and allows producers to make management decisions to increase reproductive efficiency, such as culling infertile recipients and resynchronizing recipients that are open. Knowing when cows conceive and when they will calve also helps concentrate calving supervision. Cows can be sorted into calving groups and managed to save on feed, labor, and veterinary expenses. Precalving vaccinations can be timed to ensure the most effective response. Also, avoiding overcrowding of calving pastures and/or calving cows on "fresh" pastures that have not been grazed by cows with calves has been shown to reduce calf morbidity and mortality as a result of infectious calfhood scours [62]. There are three main options to commercially diagnose pregnancy in cattle. Rectal palpation and ultrasonography are the most widely used methods for early diagnoses. Both techniques are

efficient but require a skilled technician and specific instruments and provide static information on pregnancy status at the specific moment of diagnosis. Rectal palpation is an accurate form of pregnancy diagnosis that can be performed after day 35 of gestation, whereas transrectal ultrasonography can be used to pregnancy as early as 26 days of gestation for heifers and 28 days for cows, with a high degree of accuracy [63].

Recent research has aimed to develop methods of pregnancy diagnoses that use measures of conceptus-specific markers in maternal blood as indicators of a viable pregnancy [64, 65]. After decades of research on potential biological markers of pregnancy, the measurement of pregnancy-associated glycoproteins (PAGs) in blood circulation has been established as the most reliable conceptus-derived marker for use in early pregnancy diagnosis in cattle and is commercially available for cattle producers. This test is commonly referred to as "the blood test." Produced by a specific cell population of the developing placenta, PAGs enter the maternal circulation as early as day 22–24 of gestation and reach levels currently acceptable for accurate pregnancy diagnosis at day 28. Today, commercial PAG testing is extremely accurate, providing 95–99% true positive (pregnant) reading, with false positive (reported as pregnant but actual non-pregnant) rates ranging from 1 to 5%. It is important to mention that some false positives may be due to embryonic mortality and not to test inaccuracy. Since the goal is for a producer to accurately determine non-pregnant cows, the blood tests are highly accurate and can serve as a reliable strategy to determine pregnancy in cattle. Tests currently available include BioPRYN (BioTracking LLC, Moscow, ID, USA), IDEXX Bovine pregnancy test (IDEXX Laboratories Inc., Westbrook, ME, USA), and DG29 pregnancy test (Genex Cooperative Inc., Shawano, WI USA). BioPRYN accepts blood samples from heifers 25 days post breeding and cows 28 days post breeding, IDEXX recommends day 28 blood or milk samples, and DG29 has been validated using day 29 blood samples.

Management for Efficient Recipient Utilization

Effective management of a recipient herd requires preparing the recipient to receive an embryo and identifying open recipients to be resynchronized and reused or inseminated. In any group of synchronized recipients, a small percentage will not be detected in estrus and not all detected in estrus will receive an embryo, due to either an asynchronous estrus or lack of a suitable CL at the time of transfer. If 80% of the synchronized recipients are detected in estrus and 90% of those receive embryos and 60% become pregnant, then less than 45% of any group of recipients will become pregnant. Therefore it is important to devise a strategy to resynchronize recipients as soon as possible.

In large herds with extended calving seasons, it is necessary to synchronize more than one group of recipients. Resynchronization strategies vary depending on the resources and capabilities of the ranch. With the use of ultrasonography, non-pregnant recipients may be confidently identified and resynchronized as early as three weeks after embryo transfer [66]. It is not recommended to transfer an embryo to a recipient more than twice in a given breeding season. Unpublished data from Granada BioSciences Inc. (Marquez, TX, USA) demonstrated that the pregnancy rate between recipients that became pregnant following one or two transfers was not significantly different, but less than 20% of the recipients that received a third embryo transfer became pregnant. However, a single report showed up to a 12% decrease in pregnancy rates for recipients receiving a second embryo transfer [67].

Many purebred producers contract with commercial cow-calf producers to use cows as recipients and purchase the weaned embryo transfer calf. Normally the cooperator synchronizes and observes cows for estrus, sorts recipients 6–8.5 days after estrus, and provides the labor to move the cows through the working facility for a technician to transfer embryos. The purebred producer pays the synchronization expenses and the embryo transfer technician, and pays the cow owner a premium for every embryo transfer calf weaned. This arrangement can be a win–win for both producers. However, there are issues that arise when first beginning this program, as not all commercial producers have the satisfactory management. Therefore a commercial producer already familiar with AI is a good candidate for such a program. It is essential that a producer has a good recordkeeping system, is able to weigh and identify each calf at birth, be comfortable with estrus detection, and have a good herd health program. The purebred producer may expect the cooperator to screen cows for the aforementioned diseases for biosecurity purposes.

American Embryo Transfer Association Survey

In 2011, we conducted a survey of all members of the AETA, regarded as leaders in embryo transfer of cattle in the USA. The goal of the survey was to assess the utilization of current embryo transfer practices, the perception by AETA members of each practice, and whether perceptions were supported by research. A total of 218 professional members received the survey, of whom 63 (29%) responded to the survey; 47 (75%) of the members who responded were certified AETA members, while 27 (43%) classified

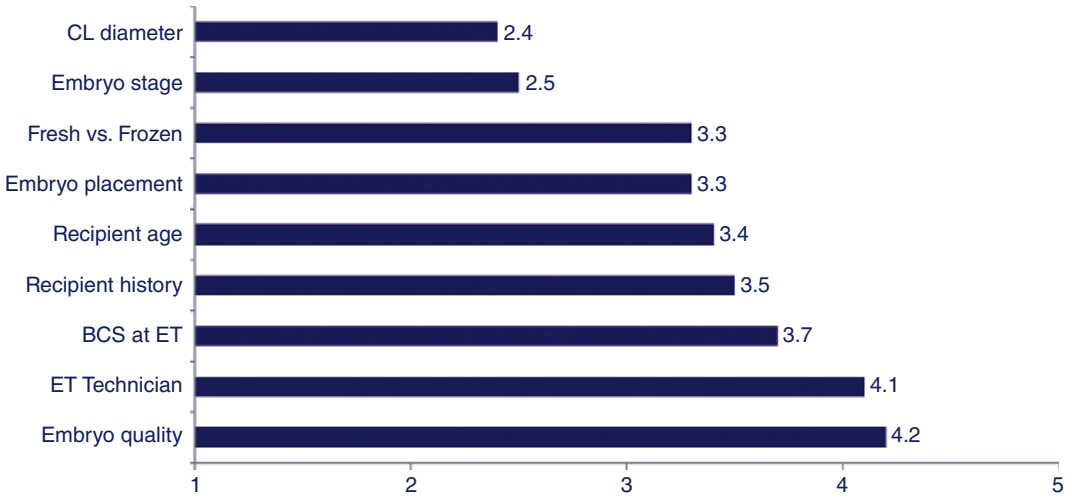


Figure 84.3 Perception of the relative impact on fertility of recipients to embryo transfer (1, least impact to 5, greatest impact). BCS, Body condition score; ET, embryo transfer.

their embryo transfer business as primarily beef cattle related, 13 (21%) as dairy cattle related, 21 (34%) as a mixture of beef and dairy, and one (2%) member was not cattle related. We asked the survey participants to rate their perception regarding several factors that could potentially affect the fertility of cows exposed to embryo transfer and the results are shown in Figure 84.3. Embryo quality was rated as the factor with the greatest impact on fertility. Quality grade of the embryo has a great impact on fertility. In a study comparing pregnancy rates of embryo transfer using embryos graded from 1 to 3 (1 being excellent and 3 being poor), recipients receiving Grade 1 embryos had greater pregnancy rates than recipients receiving Grade 3 embryos (56.1 vs 33.3%, respectively) [68]. Embryo transfer technician was rated the second most important factor affecting fertility of embryo transfer. Differences in pregnancy rates can be seen across embryo transfer technicians [69] and pregnancy rates have been inversely correlated to the time spent in the uterus during embryo transfer [54].

Recipient BCS at embryo transfer was rated of moderate impact on fertility and the effect of nutrition on the success of embryo transfer programs has been discussed in the section "Nutritional Management." Although rated as moderate, the use of fresh or frozen embryos as well as embryo stage at transfer and recipient history has a great impact on the fertility of embryo transfer programs [69]. The diameter of the CL is perceived as having a low impact on fertility of recipients of embryo transfer. In a study evaluating luteal characteristics and plasma concentrations of progesterone in pregnant and non-pregnant recipients, no differences were found in CL diameter, luteal volume, and plasma progesterone concentrations [47].

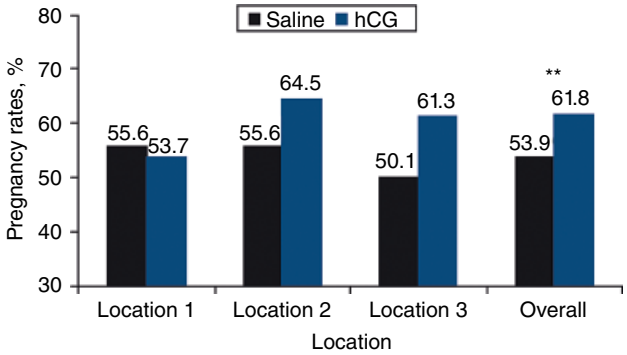


Figure 84.4 Effects of hCG 1000 IU at embryo transfer on pregnancy rates at day 33 (fresh and frozen embryos combined). **; hCG differs from saline, $P = 0.03$. *Source:* Adapted from [70].

Surprisingly, 61% of the survey participants claimed to have no experience with the use of human chorionic gonadotropin (hCG), while 23% claimed that the use of hCG is unsuccessful, and only 16% believed that the use of hCG is successful in embryo transfer programs. Nevertheless, our recent work [70] indicated that treatment with hCG at embryo transfer increased the incidence of accessory corpora lutea formation, increased progesterone in pregnant recipients, and increased transfer pregnancy rates. At three locations, purebred and crossbred Angus, Simmental, and Hereford recipients ($n = 719$) were assigned alternately to receive 1000 IU hCG intramuscularly or 1 ml saline (control) at embryo transfer. Pregnancy rates at the first diagnosis on day 33 were 61.8 and 53.9% for hCG and controls, respectively (Figure 84.4) and were 59.0 and 51.4%, respectively at the second diagnosis on day 68. In agreement with our findings, the use of hCG at embryo transfer also

increased pregnancy rates of Holstein recipient cows receiving FTET [71].

When questioned about the effects of FM on fertility of embryo transfer recipients, 53% of the survey participants claimed that the use of FM was not successful, 18% claimed to have no experience with FM, and only 29% rated the use of FM as successful in embryo transfer programs. In a study evaluating the effects of FM prior to embryo transfer, pregnancy rates were greater for recipients receiving an injection of FM. However, the results varied by location [43]. In another study, FM treatment improved overall pregnancy rates of recipients (65.3 vs 60.2% for FM and control, respectively) and the effects of FM appeared to be even more beneficial when transferring Grade 2 embryos [72].

Finally, we asked participants for their perception of the success of FTET programs; 73% claimed that FTET is successful, while 14% claimed that FTET is unsuccessful, and 13% had no experience with FTET. The use of synchronization protocols excludes the need for estrus detection and increases the number of animals receiving embryos. Most current FTET protocols are based on progestin-releasing devices combined with estradiol or GnRH, which control and synchronize follicular wave dynamics and ovulation. Conception rates to a single FTET have been reported to be similar to those after detection of estrus; however, pregnancy rates are higher because these treatments have increased the proportion of recipients that receive an embryo [68].

Conclusion

For an embryo transfer program to be effective, numerous factors need to be put in place to ensure success. Nutrition, estrous cycle control, and recipient management are all responsible for the success or failure in a given program. Producers, embryologists, veterinarians, and all members of the recipient management team need to be aware of the short- and long-term factors that contribute to recipients conceiving to embryo transfer, maintaining the embryo/fetus to term, delivering the calf without assistance, and raising and weaning a healthy calf.

Summary

A commercially viable cattle embryo transfer industry was established during the early 1970s. Initially, techniques for transferring cattle embryos were exclusively surgical. However, by the early 1980s, most embryos were transferred non-surgically. For an embryo transfer program to be effective, numerous factors need to be put in place to ensure success. Nutrition, estrous cycle control, and recipient management are all responsible for the success or failure in fertility for a given herd. Utilization of BCS is a practical method for determining nutritional status of the recipient herd. Prepartum nutrition is critical to ensure that cows calve in adequate body condition to reinitiate postpartum estrous cycles early enough to respond to synchronization protocols. Estrus synchronization for embryo transfer after detected estrus or for FTET are effective methods for increasing the number of calves produced by embryo transfer. In addition, resynchronization of non-pregnant females effectively ensures that a high percentage of recipients will return to estrus during a 72-hour interval and are eligible for subsequent embryo transfers. Many components need to be assessed to ensure that the recipient herd achieves its reproductive potential. These factors include assessing the merits of nulliparous, primiparous, or multiparous cows, ensuring that facilities allow for minimal stress, and that the herd health program is well defined and followed. Numerous short- and long-term factors contribute to recipients conceiving to a transferred embryo, maintaining the embryo/fetus to term, delivering the calf without assistance, and raising and weaning a healthy calf.

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85

Evaluation of *In Vivo* Derived Bovine Embryos

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Introduction

Morphological evaluation of embryo quality is performed for three major reasons. The first reason is to differentiate between embryos and unfertilized ova. Careful evaluation of harvested embryos/ova is necessary to ensure that viable embryos are not discarded or, conversely, that unfertilized ova are not transferred or cryopreserved. The second reason is to determine if the developmental stage of the embryos is consistent with the expected developmental stage based on the days since estrus that the embryos were collected. There is often a high degree of variability in observed stage of embryonic development among embryos obtained during embryo recovery from a single donor female, and this variation in stage of development should be considered when selecting a suitable recipient female. The third reason is to enable technicians to have sufficient information on which to base the decision to transfer or to cryopreserve the harvested embryos. Poorer quality embryos that may result in pregnancies if transferred fresh usually do not survive the cryopreservation process.

Despite the importance of this topic, embryo evaluation is a difficult aspect of the overall embryo transfer process to learn, especially if attempting to self-train. Although this chapter will provide numerous helpful photographs of embryos and unfertilized ova, beginners would benefit from joining an experienced embryo transfer mentor for intensive and repetitive training sessions on morphological embryo evaluation. Proper training, proper equipment, and extensive experience are the three major factors affecting one's proficiency with embryo evaluation.

History of Bovine Embryo Evaluation

The very earliest stages of bovine embryonic development (the ovum and 2-cell embryo) were first described in 1931 by Hartman et al [1]. In the following decade two other research groups [2, 3] described and illustrated various stages of bovine embryonic development. These very early descriptions of embryonic development served as the foundation of the morphological embryo evaluation method that subsequently evolved and became widely used in the commercial bovine embryo transfer industry [4].

The morphological embryo evaluation method depends on the human eye to critically evaluate subtle differences among harvested embryos. During the early years of its use, the method was criticized as being too subjective, inconsistent, and prone to error. As a result, several alternative methods for assessing embryo quality were investigated for their potential to be less subjective and more consistent than morphological evaluation. Some of these methods included the ability of an embryo to continue its development during *in vitro* culture [5, 6], the dye exclusion test [7], measurement of embryonic metabolic activity [6], glucose uptake [8], live–dead stains [9], computerized image analysis [10], fluorescein diacetate vital stain [11, 12], DAPI (4′,6′-diamidino-2-phenyl-indole) staining for dead cells [13], and *in vivo* culture in rabbit oviducts [14].

Refinement of the Morphological Embryo Evaluation Method

Despite the numerous attempts to develop a more precise and predictable method to evaluate the viability of bovine

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

embryos, no methods have been devised that are considered better or more accurate than morphological embryo evaluation. This method is the preferred method of embryo viability assessment under clinical conditions because it is a relatively simple and fairly rapid technique for evaluation of bovine embryos.

After the early report on bovine embryo evaluation in 1976 by Shea et al. [4], three other landmark papers on embryo evaluation were subsequently published [15–17]. These practitioners and scientists described the criteria that are, for the most part, still used today as a general guideline for embryo evaluation and prediction of potential pregnancy. Linder and Wright [17] described numerous parameters commonly used for embryo evaluation, including stage of embryonic development and structural features such as embryo size and shape, presence of extruded cells or degenerated cells, color characteristic, number and compactness of the cells (also called blastomeres) that comprise the early embryo, integrity of the zona pellucida, and presence or absence of vacuoles within the cytoplasm of the embryonic cells.

History of the IETS Grading and Classification System

The International Embryo Technology Society (IETS) is an international organization that was born in 1974 in Denver, Colorado, USA. It was created by a group of US practitioners and scientists who recognized the need to share scientific discoveries. As the worldwide commercial embryo transfer industry grew, it became critical to develop standardized embryo nomenclature so that buyers and sellers of embryos knew precisely the stage and quality of embryos being marketed.

The IETS published in their manual [18] a recommended two-digit coding system to uniformly and systematically describe embryos and their characteristics. On completion of the morphological assessment of an embryo, it is assigned two numbers, separated by a hyphen, that correspond to the embryonic stage of development and embryo quality grade. The standardized coding system used to describe the stage of embryonic development ranges from 1 (an unfertilized oocyte) to 9 (expanding hatched blastocyst). The standardized code for embryo quality, based on morphological characteristics of embryos, is also numerical and ranges from 1 (excellent/good) to 4 (dead/degenerating). This IETS embryo grading and classification system must be used for international embryo trade, and because of this mandate most practitioners have adopted this system for everyday use. A more detailed description of the IETS embryo grading and classification system is discussed later in this chapter (see section "IETS Grading and Classification System").

The Importance of a Good Microscope

Before delving into the specific methodology used for morphological evaluation of embryos, it is important to remind readers that proper equipment is needed for accurate embryo evaluation. Although many beginners in embryo transfer are tempted to purchase an inexpensive microscope as a means to reduce start-up costs, doing so will likely create difficulties in finding and evaluating embryos. A poor-quality microscope can make it extremely challenging for a practitioner to tell the difference between an unfertilized ovum (UFO) and a compact morula. Similarly, a practitioner will be hampered by a poor-quality microscope when attempting to discern the various embryo quality grades.

Unlike semen evaluation, where a compound microscope is used, embryo transfer requires the use of a stereomicroscope. Stereomicroscopes, also called dissecting microscopes, are designed for use with three-dimensional specimens. Preimplantation bovine embryos, although microscopic in size, are round like a basketball and thus are best evaluated using a stereomicroscope.

There are several important features of a stereomicroscope that can directly influence one's ability to easily and accurately evaluate embryos. One important feature is the overall magnification. Overall magnification of a stereomicroscope is a function of the magnification of the eyepieces, objective lens, and zoom changer. For example, total magnification of a stereomicroscope equipped with 10× eyepieces, a 1× objective lens, and a 2× zoom changer would be approximately 20× ($10 \times 1 \times 2 = 20$). Many technicians prefer to search for embryos at a total magnification between 10× and 15×, but to effectively evaluate the fine details of an embryo and to identify any abnormalities, such as cracks in the zona pellucida, embryos should be observed under a good optical quality stereomicroscope with a magnification range that reaches at least 50×. It should be noted that the IETS-approved procedure for sanitary handling of embryos requires that embryos be inspected at a magnification of at least 50×.

Most embryo transfer technicians prefer that the eyepieces not exceed 10× in order to maintain an adequate field of view and image resolution. A microscope equipped with a diopter adjustment on one eyepiece (if not both) is very useful for compensating for differences in eyesight between a technician's left and right eyes. An adjustable binocular tube will also enable multiple technicians with varying interpupillary distances to use the same stereomicroscope.

A second important feature of a stereomicroscope for embryo handling and evaluation is the working distance (distance from the embryo to the objective lens). The working distance should be long to give maximal flexibility when searching for embryos, and the microscope stage should be large and free of obstacles such as stage clips, power buttons, or similar items that make sliding of Petri dishes on the stage difficult. A large and clear stage is important for providing adequate space to move Petri dishes and to operate the embryo handling device without accidently spilling the Petri dish and losing the embryos.

A third important feature of a stereomicroscope used for embryo evaluation is the illumination. The illumination should come from underneath the stage plate and it should be bright and uniform across the entire stage plate. A clear stage plate is needed; frosted stage plates are not suitable for embryo work. Brightfield illumination is standard for most stereomicroscopes, but darkfield illumination can be helpful when searching through "cloudy" fluids in a search dish.

Normal *In Vivo* Bovine Embryonic Development

In order to know whether embryos harvested from a donor have developed at the expected rate, it is important for technicians to have a good understanding of normal *in vivo* bovine embryonic development. The overall diameter of a bovine embryo is 150–190 µm (or about 15 times the size of white blood cells), which includes the zona pellucida thickness of approximately 12–15 µm [17]. The overall diameter of the zona pellucida-enclosed embryo remains virtually unchanged from the 1-cell stage of development until the blastocyst stage of development because blastomeres (individual cells of an embryo prior to cellular differentiation at the blastocyst stage of development) become progressively smaller with each cell division.

The zona pellucida is a translucent shell surrounding an embryo from the 1-cell until the expanded blastocyst stage of development. The zona pellucida functions as a physical barrier against pathogens, contains receptors for sperm, and blocks accessory sperm from reaching the ovum. Once an embryo reaches the expanded blastocyst and continues to grow, the embryo becomes so large that it ruptures the zona pellucida and "hatches."

During normal *in vivo* embryonic development, blastomeres go through a series of cleavage divisions. The ovum, after fertilization, divides and develops into a 2-cell embryo, the 2-cell develops into a 4-cell, the 4-cell into an 8-cell, and so on. When the blastomeres appear like a cluster of grapes and individual blastomeres cannot be differentiated, this stage of embryonic development is known as the morula stage. As the embryo further develops, it prepares to undergo its first differentiation event known as blastulation. Just prior to this differentiation event, cells of the morula "compact" and are allocated to the "inside" and "outside" parts of the embryo.

Once blastulation has occurred, the embryo is at the blastocyst stage of embryonic development. The blastocyst contains two populations of differentiated cells: an outer ring of trophoblast cells and an inner clump of cells known as the inner cell mass (ICM). The ICM will give rise to the fetus and most layers of the placenta, while the trophoblast cells will eventually form the outermost layer of the placenta. The blastocyst stage of embryonic development has another distinguishing feature, the presence of a blastocoele cavity. This fluid-filled cavity is surrounded by the trophoblast cells and grows progressively larger to help cause thinning of the zona pellucida prior to embryo hatching. Figure 85.1 illustrates the features observed in a blastocyst-stage bovine embryo, while Figure 85.2 shows the stages of bovine embryonic development.

IETS Grading and Classification System

Embryos are typically recovered six to eight days after the onset of estrus. Harvested embryos should be classified into groups based on their stage of embryonic development and quality grade. Table 85.1 illustrates the current IETS embryo evaluation system followed by a detailed description of these developmental stages. After a technician completes the embryo evaluation process, a two-digit code is assigned to each embryo. For example, a fair-quality early blastocyst would be denoted as 5-2. A blastocyst-stage embryo of excellent or good quality would be denoted as 6-1.

Stage of Embryonic Development

Stage Code 1 : 1-Cell

For any entity recovered on days 6–8 that is truly only a single cell, it is safe to assume that it is a UFO. However, a

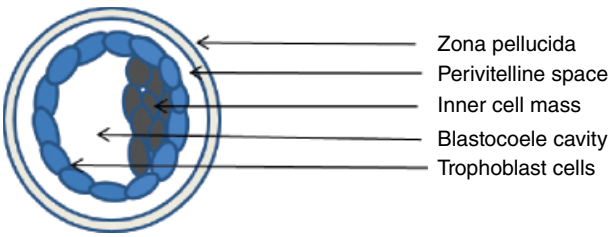


Figure 85.1 A blastocyst-stage bovine embryo.

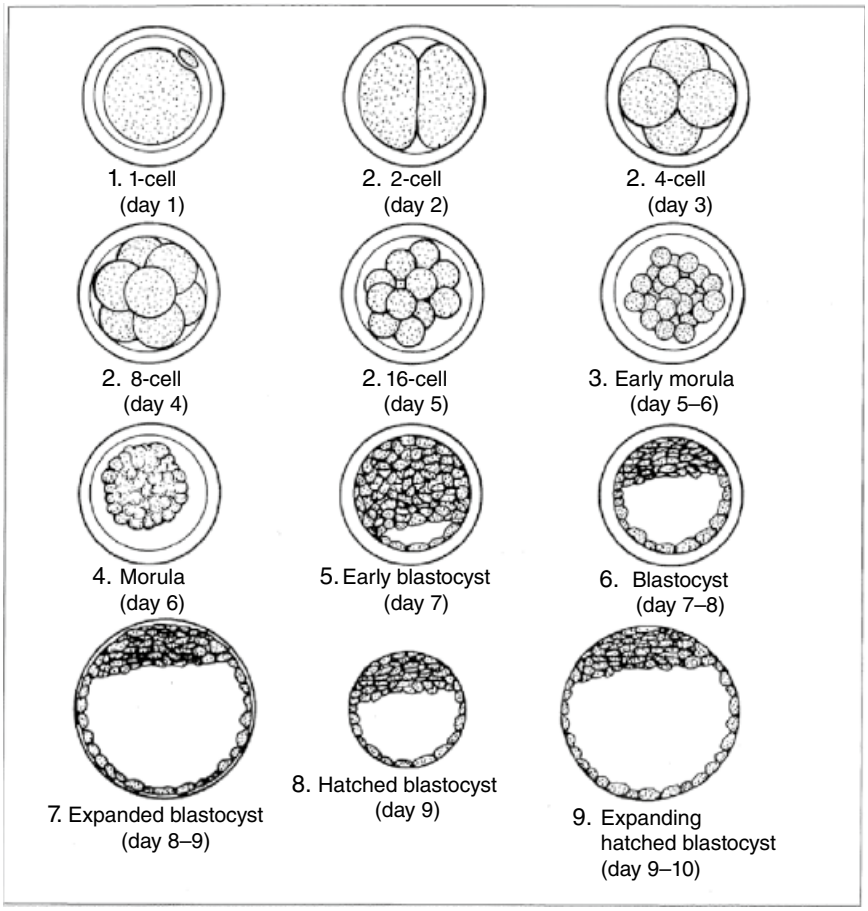


Figure 85.2 Normal embryonic development of bovine embryos. *Source:* From [19], © 2010, IETS.

Table 85.1 IETS recommended codes for embryonic stage of development and embryo quality grade.

Stage of embryonic development		Quality grade of embryo	
Code	**Stage**	**Code**	**Quality description**
1	Unfertilized	1	Excellent or good
2	2-cell to 12-cell	2	Fair
3	Early morula	3	Poor
4	Morula	4	Dead or degenerating
5	Early blastocyst		
6	Blastocyst		
7	Expanded blastocyst		
8	Hatched blastocyst		
9	Expanded hatched blastocyst		

challenging task for an inexperienced technician is to distinguish a UFO from a compact morula (stage code 4, see below). Discerning a UFO from other stages of embryonic development can also be difficult because not all UFOs have the same morphological appearance. Many "normal" UFOs have a perfectly spherical zona pellucida, perfectly spherical oolemma (also called the vitelline membrane), relatively uniform granularity in the cytoplasm, and a moderate volume of perivitelline space. However, other UFOs may show varying signs of degeneration including cytoplasmic fragmentation, which can lead to the illusion of a blastocoele cavity and/or appearance of cell division, and extreme condensation, which can lead to confusion with a compact morula.

Stage Code 2 : 2-Cell to 12-Cell

Cells of a preimplantation embryo prior to morphological and functional differentiation are called blastomeres. Any embryo recovered six to eight days after the onset of estrus

that contains 2–12 blastomeres is almost certainly dead or degenerate. Under normal conditions this stage of embryonic development is expected to be present in the oviduct (not the uterus) prior to day 5 of the bovine estrous cycle (day 0 = onset of estrus). Clearly, if a 2- to 12-cell embryo were somehow still viable, its development is severely delayed and it should not be considered for transfer or cryopreservation.

Stage Code 3: Early Morula

The term "morula" has its origin in the Latin word for mulberry. Embryos at the morula stage of embryonic development consist of a group of at least 16 cells. Although some individual blastomeres are easy to visualize, others are only partially visible as they are "hidden" by other blastomeres. This stage of embryonic development, like all stages of development discussed previously, is not well suited for cryopreservation.

Stage Code 4: Compact Morula

Individual blastomeres present in this embryo have coalesced to form a tightly compacted ball of cells. It is impossible to discern individual blastomeres at this stage of embryonic development. As a part of the compaction process, cells are allocated to the "inside" and also to the "outside" part of the ball of cells. The cells on the outside of the ball form tight junctions with one another in a contiguous ring. The cells comprising the embryo occupy approximately 60–70% of the space inside the zona pellucida (called the perivitelline space). As stated earlier, it is this stage of embryonic development that can be easily confused with a UFO.

Stage Code 5: Early Blastocyst

The most distinguishing characteristic of an early blastocyst-stage embryo is the presence of a small fluid-filled cavity known as the blastocoele. Several differentiated trophoblast cells will also be visible between the blastocoele cavity and the zona pellucida, although the ability to distinguish them from the cells of the ICM is quite limited at this stage. The cells comprising the embryo occupy approximately 70–80% of the perivitelline space. The zona pellucida is still the same thickness as the less developmentally advanced stages of embryonic development previously discussed.

Stage Code 6: Blastocyst

A blastocyst-stage embryo will have a clearly defined trophoblast layer, blastocoele cavity, and group of ICM cells, along with a normal-thickness zona pellucida. There will be little to no perivitelline space present except in cases where the embryo may be partially collapsed.

Stage Code 7: Expanded Blastocyst

The most distinguishing feature of an expanded blastocyst-stage embryo is an increase in its overall diameter, 1.2–1.5 times larger than its original diameter of 150–190 μm, coupled with a thinning of the zona pellucida to approximately one-third of its original thickness of 12–15 μm. Because of the large increase in the volume of the blastocoele cavity at this stage of embryonic development, no perivitelline space is present. Interestingly, expanded blastocyst-stage embryos frequently appear collapsed. This phenomenon is characterized by a partial loss of blastocoele cavity fluid and is believed to be a normal phenomenon that contributes to embryo hatching. The zona pellucida, once thinned, never regains its original thickness.

Stage Code 8: Hatched Blastocyst

Hatched blastocyst-stage embryos can be undergoing the process of hatching or may have completely hatched from the zona pellucida. Embryos at this stage may be spherical with a well-defined blastocele cavity or may be collapsed. Identification of this stage of embryonic development can be quite difficult if the embryo has completely hatched from the zona pellucida and has nearly totally collapsed because it can easily be mistaken for a piece of endometrial tissue. This stage of embryonic development is not eligible for international trade because the non-intact zona pellucida makes it impossible to wash the embryo in accordance with IETS procedures for sanitary handling of embryos [20].

Stage Code 9: Expanded Hatched Blastocyst

This stage of embryonic development is identical in appearance to a completely hatched stage code 8, except for being significantly larger in diameter. It is uncommon to recover expanded hatched blastocysts from bovine donor females unless flushing is performed more than eight days after the onset of estrus. This stage of embryonic development is not eligible for international trade.

Embryo Quality Grade

Embryo quality grade is determined by visual assessment of an embryo's morphological characteristics. Characteristics used to determine the quality grade of an individual embryo include uniformity of blastomeres (size,

color, and shape), presence of extruded cells, presence of dead/degenerating blastomeres, degree of cytoplasmic granularity, presence of cytoplasmic vacuoles, cytoplasmic fragmentation, and structural integrity of the zona pellucida.

Quality Grade Code 1: Excellent or Good

A quality grade 1 embryo has a symmetrical and spherical embryo mass with individual cells that are uniform in size, color, and density. The embryonic stage of development is consistent with that expected based on the day of the donor female's estrous cycle when the embryo was recovered. Irregularities (such as cytoplasmic vacuoles), if any, are relativity minor, and at least 85% of the embryonic cells are an intact, viable, and cohesive mass (i.e. no more than 15% of embryonic cells are extruded into the perivitelline space). The zona pellucida should be spherically shaped and smooth, with no concave or flat surfaces that might cause the embryo to adhere to a Petri dish or straw.

Quality Grade Code 2: Fair

Moderate irregularities exist in the overall shape of the embryonic mass or in the size, color, and density of individual cells comprising the embryo. Mild unevenness of cytoplasmic pigmentation within individual blastomeres (do not confuse this with translucency of the blastocoele cavity and darkness of the ICM cells of a blastocyst-stage embryo), "peppering" of cytoplasm within individual blastomeres, and/or a small number of vacuoles within the cytoplasm of blastomeres would be sufficient to warrant a quality grade 2 score. At least 50% of the embryonic cells should be an intact, viable, and cohesive embryonic mass.

Quality Grade Code 3: Poor

Major irregularities exist in the shape of the embryonic mass or in the size, color, and density of individual cells. Extreme unevenness of cytoplasmic pigmentation within individual blastomeres, significant "peppering" of cytoplasm within individual blastomeres, and/or large numbers of vacuoles within individual blastomeres are sufficient to warrant a quality grade 3 score. At least 25% of the embryonic cells should be an intact, viable, and cohesive embryonic mass. Multiple extruded cells of widely varying sizes, indicating a prolonged and persistent problem with the cohesiveness of embryonic blastomeres, would cause an embryo to be assigned a quality grade 3 score.

Quality Grade Code 4: Dead or Degenerating

Entities that are assigned a quality grade 4 score are nonviable and should not be transferred to recipient females or cryopreserved. Dead/degenerating embryos typically fall in this category because of extremely dark cytoplasm, nonintact cell membranes, and other significant defects. Because embryos typically are recovered on approximately day 7 of the estrous cycle, any embryo that has not advanced to at least the morula stage of embryonic development should be assigned an embryo quality grade 4 score.

Despite the strong correlation that exists between embryo quality grade and post-transfer pregnancy rate, some quality grade 1 embryos fail to produce a pregnancy after transfer, whereas some quality grade 3 embryos produce a higher than expected pregnancy rate. The *IETS Manual* [19] states:

> It should be recognized that visual evaluation of embryos is a subjective valuation of a biological system and is not an exact science. Furthermore, there are other factors such as environmental conditions, recipient quality, and technician capacity that are important in obtaining pregnancies after transfer of embryos.

Embryo Evaluation Procedure

During the recovery procedure, embryos are typically collected using an embryo filtration device that minimizes the volume of flushing medium through which the technician must search to locate the embryos. Some embryo filtration devices also serve as the embryo search dish, whereas other devices require that embryos be transferred from the filtration device to a Petri dish for searching. After embryos are recovered, they must be located in the recovery medium using a stereomicroscope typically under 10–15× magnification. Embryos are subsequently transferred to a Petri dish containing embryo holding medium, where they will be evaluated at a minimum magnification of 50× before being assigned the two-digit IETS code for stage of development and embryo quality.

As illustrated in Figure 85.2, the compact morula, early blastocyst, and blastocyst stages of embryonic development are the ones most commonly recovered on day 7 after estrus. The expanded blastocyst is typically recovered on day 8 but can sometimes be found after a day-7 embryo recovery. The zona pellucida, because of its translucency and ability to refract light, is the landmark structure that helps technicians identify embryos and ova during the search procedure. Once an embryo progresses to the

hatched blastocyst stage it may become harder to identify because it may have a collapsed appearance and, without the zona pellucida, it can easily be mistaken for endometrial cells or other cellular debris in the Petri dish.

Although each harvested embryo must be assigned its own two-digit code, there is great value in examining all harvested embryos/ova as a group. This comparative evaluation usually makes it easy for the technician to distinguish UFOs from embryos, as well as to compare embryos that are expanded and have a thinner zona pellucida with embryos that have not yet expanded.

A good predictor of an embryo's viability is its stage of development relative to what it should be on a given day after estrus. From a superovulated cow, one should expect variety in the stages of embryonic development recovered because there is variation in the time at which ovarian follicles rupture and release their ova. Similarly, one should also expect variation in embryo quality grade. It is not uncommon to obtain from the same donor female excellent quality embryos in addition to UFOs and degenerate embryos. Based on the American Embryo Transfer Association (AETA) survey report [21], the average number of viable embryos recovered from dairy donors was 5.7 (representing 56% of the total ova/embryos recovered) and from beef donors was 6.9 (also representing 56% of total ova/embryos recovered). The average number of degenerate embryos and unfertilized ova recovered was 1.6 (14% of total embryos/ova) and 3.1 (29% of total embryos/ova), respectively, from dairy donors and 2.0 (15% of total ova/embryos) and 3.5 (27% of total ova/embryos), respectively, from beef donors.

Practical Application of Embryo Evaluation Guidelines

To help technicians learn how to properly evaluate bovine *in vivo* derived embryos, Figure 85.3 depicts (using photographs of actual bovine ova and embryos) the range in embryonic developmental stage that may be obtained after performing a day-7 embryo recovery. A more developmentally advanced embryo (expanded hatched blastocyst) is shown in Figure 85.3b, although an embryo at this developmental stage is not usually expected on day 7. Many people find the side-by-side photos of actual specimens more helpful for learning embryo grading and classification than illustrations/sketches. In addition to the composite image in Figure 85.3 depicting the range in embryonic developmental stages, the figures in the remainder of this chapter show not only the principles of embryo grading and classification but also some of the common challenges faced by embryo technicians.

One important point to make before proceeding further is that although each stage of embryonic development is assigned a specific code, embryos are continually developing and are gradually transitioning from one stage of development to the next. As a result, many embryos may actually be between two different developmental stages at the time of recovery. Any time an embryo is making the transition from one stage to the next, it increases the likelihood that it may be called one developmental stage by one technician and a different developmental stage by another technician (e.g. stage 4 by some and stage 5 by others).

The following discussion of embryo evaluation is divided into sections on non-transferable quality embryos and transferable quality embryos.

Non-Transferable Quality Embryos

During the initial phase of the embryo evaluation process, most technicians segregate non-viable (non-transferable quality) ova/embryos from viable (transferable quality) embryos. Obviously, non-viable embryos should not be transferred to recipient females nor should they be cryopreserved.

Unfertilized Ova

Unfertilized ova collected six to eight days after the onset of estrus can have many different physical appearances (Figure 85.4). The three major keys to identifying a UFO are (i) the vitelline membrane (also called the oolemma) that surrounds the cytoplasm of the ovum is smooth and nearly perfectly spherical unless the ovum is fragmented or degenerate; (ii) the cytoplasm is granular and not cellular in appearance; and (iii) there is a moderate amount of perivitelline space (the space between the ovum and the zona pellucida), except in cases of degeneration.

As alluded to above, it can be frustrating to definitely determine the stage of development of ova that have undergone degeneration or fragmentation. Degenerate ova can, on occasion, appear very similar to a compact morula. Fragmentation, sometimes referred to as cytoplasmic blebbing, is a process during which some of the cytoplasm (which does not contain any chromosomes or chromosomal DNA) segregates itself from the ovum, resulting in a specimen that appears to be multicellular. Typically, the structure formed as a result of fragmentation is far smaller in size than would be expected for a blastomere of a 2-cell or 4-cell embryo. However, unless a fragmented ovum is exposed to a DNA-specific stain (e.g. bis-benzimide), one cannot determine with certainty if the entity is a stage code 2 embryo or a fragmented ovum. From a very practical

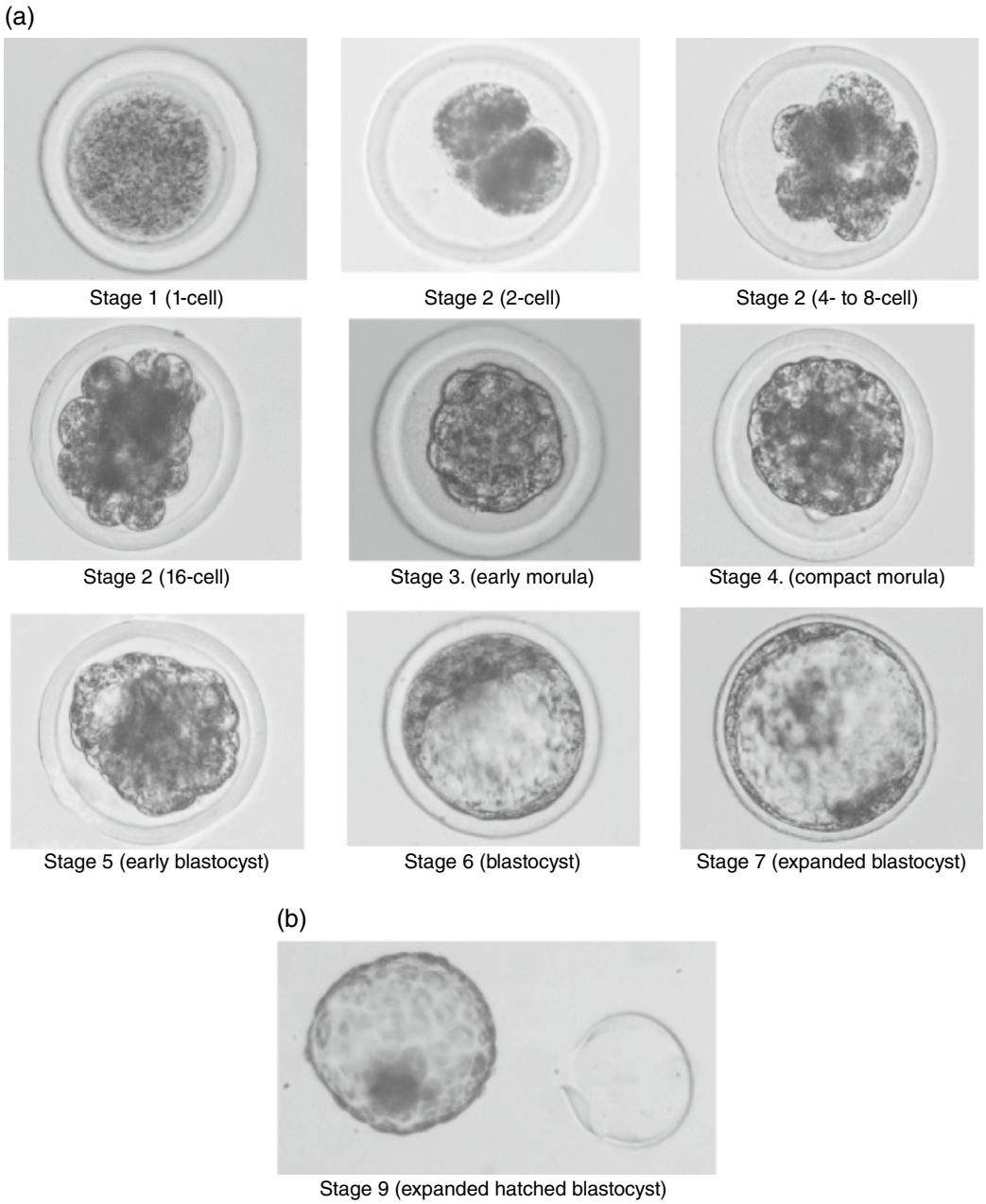


Figure 85.3 (a) Comparison of developmental stages of *in vivo* derived bovine embryos collected on days 6–8 after estrus. At day 7, a stage code 1 embryo is considered an unfertilized ovum and a stage code 2 embryo is considered dead or degenerate. (b) Expanded hatched blastocyst typically recovered on or after day 8 after estrus; note the empty zona pellucida on the right and the expanded embryo on the left. *Source:* Image b courtesy of Dr. Brad Lindsey.

perspective, however, the distinction between stage code 1 and stage code 2 is irrelevant because neither is suitable for transfer.

Dead or Degenerating Embryos

If embryos at stage code 2 are collected on day 7, the stage of embryonic development is not consistent with the expected stage of development. Therefore these embryos are classified as degenerate (quality grade 4) because they are severely retarded in their development. Degenerate embryos are ones that have been fertilized but which died some time during the developmental process.

An embryo could also be considered dead or degenerate if its blastomeres are not fused to one another (i.e. there is lack of tight cell junction formation between the cells of the embryo). Failure of blastomeres to develop in a tight and cohesive group, to compact, and to form tight cell

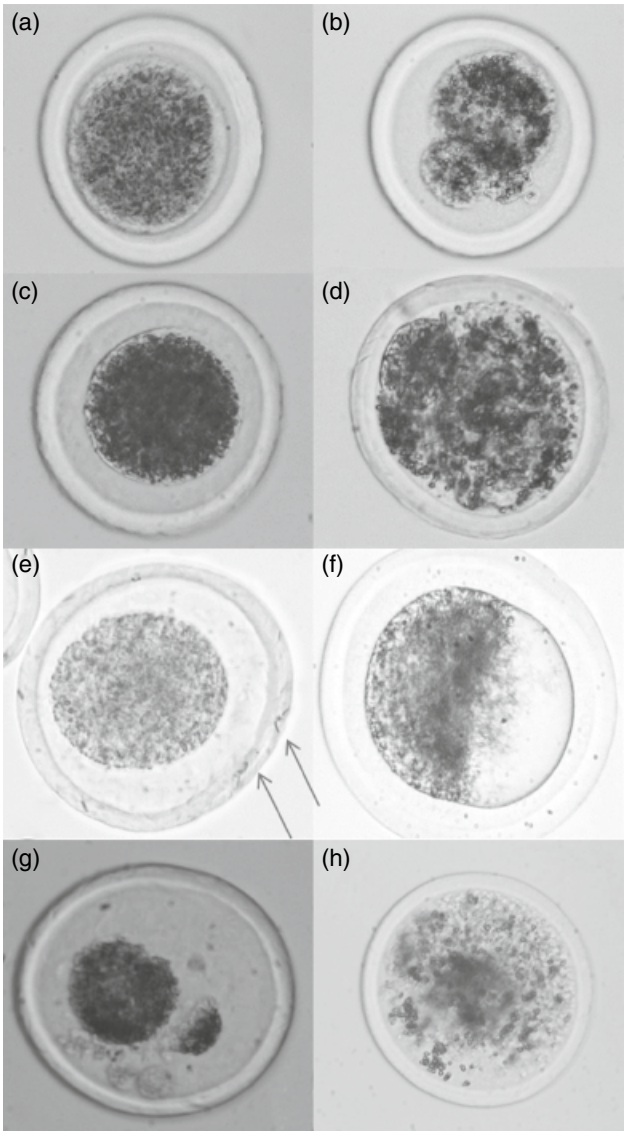


Figure 85.4 Unfertilized ova recovered non-surgically from superovulated donor cows six to eight days after the onset of estrus. Note the extremely wide variation in morphology which makes accurate identification difficult for inexperienced embryo technicians. (a) Unfertilized ovum with smooth vitelline membrane and granular cytoplasm that can be mistaken for a compact morula. (b) Fragmented unfertilized ovum. (c) Unfertilized ovum possessing dark cytoplasm and a smooth vitelline membrane; can be mistaken for a compact morula. (d) Degenerate unfertilized ovum. (e) Unfertilized ovum with light-colored granular cytoplasm; note sperm (arrows) attached to the zona pellucida. (f) Unfertilized ovum with smooth vitelline membrane; ovum is granular in one half and clear in the other half; can be mistaken for a blastocyst-stage embryo. (g) Fragmented unfertilized ovum. (h) Degenerate unfertilized ovum; note "loss" of vitelline membrane.

junctions is a sign that the embryo is in the process of degenerating or dying (Figures 85.5 and 85.6). Note in Figure 85.6 a compact morula (a) next to a degenerate/dead embryo (b).

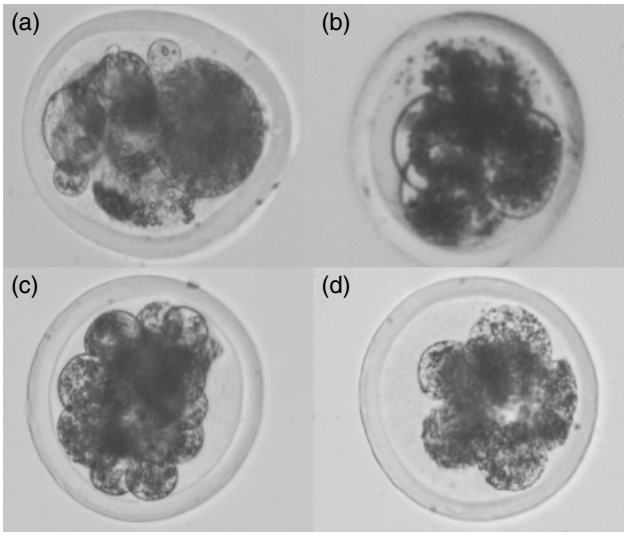


Figure 85.5 Degenerate bovine embryos collected on day 7: (a) and (b) are degenerate and are considered dead; (c) and (d) are not as developmentally advanced as expected for a day-7 embryo, and the cells are not fused tightly to one another, so are also considered degenerate.

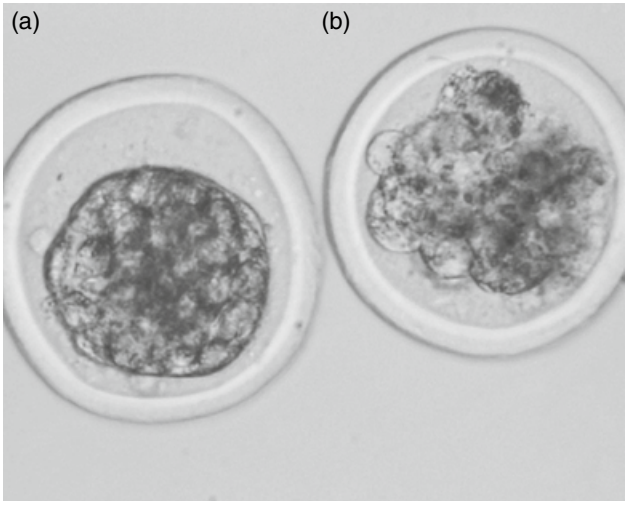


Figure 85.6 Day-7 compact morula (a) adjacent to a degenerate embryo (b).

Transferable Quality Embryos

Once a technician has discarded UFOs and non-transferable quality embryos, attention should be directed to the remaining embryos that are of transferable quality. In a perfect world, all transferable quality embryos would be quality grade 1. However, this is not the case. There are a number of "defects" in transferable quality embryos that can cause their embryo quality grade to be lowered to a quality grade 2 or 3. These "defects" are discussed in some detail in the following text.

What are the characteristics of a quality grade 1 embryo? In essence, it is the lack of "defects" that would lead to assignment of a quality grade 1. Although a number of quality grade 1 embryos have already been shown (e.g. Figures 85.3 and 85.6), the main emphasis in the remaining discussion will be on identification of the embryo "defects" that lead to assignment of lower quality grade scores. In many photographs, however, quality grade 1 embryos will be shown side by side with quality grade 2 and 3 embryos, as well as UFOs, for easy comparison.

A number of morphological characteristics of an embryo can be considered as a deviation from normal. These abnormalities can include irregular sizes of embryonic blastomeres, large and/or multiple vacuoles within the cytoplasm of embryonic cells, degeneration of one or more cells in the embryo, some cells not adhering to the other cells comprising the embryo (i.e. extruded blastomeres/cells), and a damaged or misshapen zona pellucida. Many viable embryos have some detectable morphological abnormality such as a small number of extruded cells. Embryo quality grading must consider not only the presence or absence of these abnormalities but also the extent/degree to which these deviations are expected to influence post-transfer embryo survival. Experience has shown, for example, that an embryo that possesses a few degenerate cells but which consists mostly of normal appearing cells has an excellent chance of establishing a pregnancy and producing a live calf.

Extruded Cells

One abnormality that occurs somewhat frequently during preimplantation embryonic development is failure of all cells of an embryo to continue developing. Ordinarily, all cells of an embryo adhere to and communicate with one another. However, in certain cases some cells do not participate with their cohorts in normal embryonic development. The cells that do not fully participate in embryonic development are known as extruded cells because they are not adhered to the rest of the embryo and are found in the perivitelline space. Extruded cells often detract from the developmental potential of an embryo, particularly when the cells are extruded early in the developmental process. Thus, the size and number of extruded cells directly impacts embryo quality grade.

Extruded cells typically do not divide, and the extrusion of cells from an embryo can occur at any developmental stage beginning at the 2-cell stage. As long as a substantial number of blastomeres within an embryo remain healthy, the embryo can produce a viable pregnancy even when extruded cells are present. The rate of pregnancy depends on the number and size of extruded cells. Several examples of embryos possessing extruded cells are shown in Figures 85.7–85.11. It should be noted that extruded cells in stage code 6 and 7 embryos get pressed against the zona pellucida and may not be obvious (Figure 85.11) or may lead to assignment of a lower than deserved quality grade score (Figure 85.9). Embryos should be rolled in the Petri dish to enable views from multiple angles.

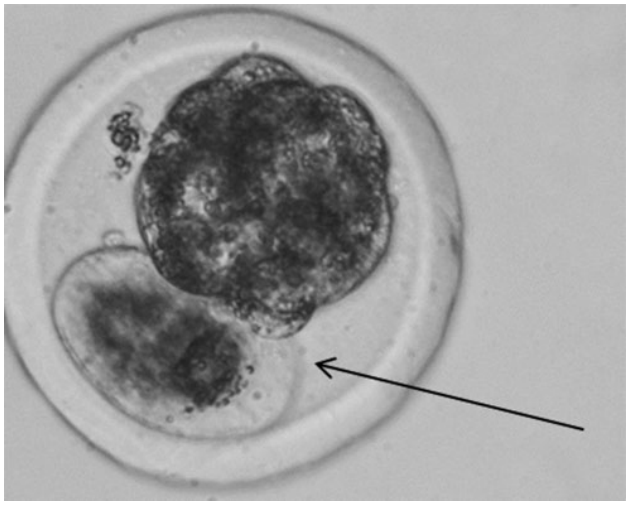


Figure 85.7 Grade 2 compact morula. Note one large blastomere (arrow) has stopped dividing, while others have formed a compact morula.

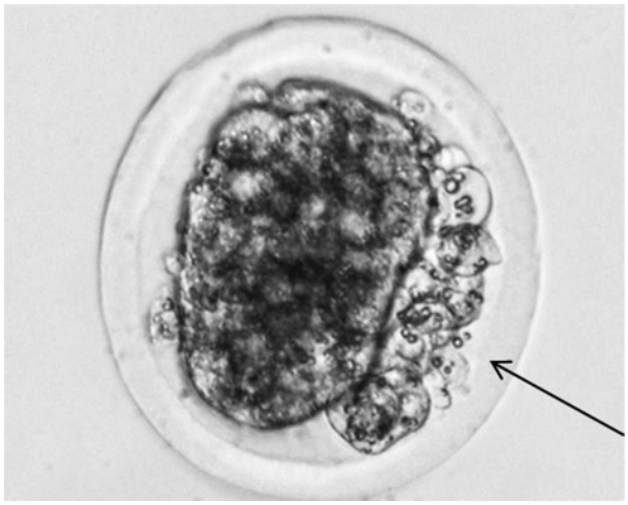


Figure 85.8 Grade 2 morula. Note several extruded blastomeres (arrow) to the right side of the embryo.

Misshapen or Flat Zona Pellucida

A zona pellucida is considered misshapen if it is not spherical or has a flat or concave surface that can cause the embryo to stick to a Petri dish or straw. This defect, by itself,

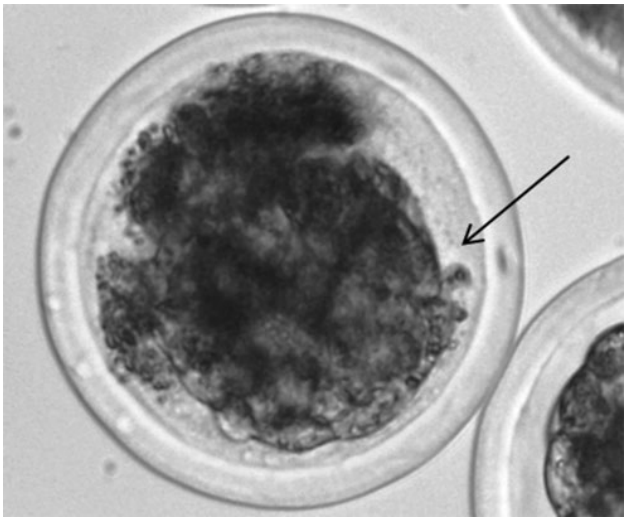


Figure 85.9 Grade 3 morula. Note embryonic cell mass on the right side (arrow) hidden by a large number of extruded cells in the upper left corner and left side. May be called grade 4 by some without rolling this embryo.

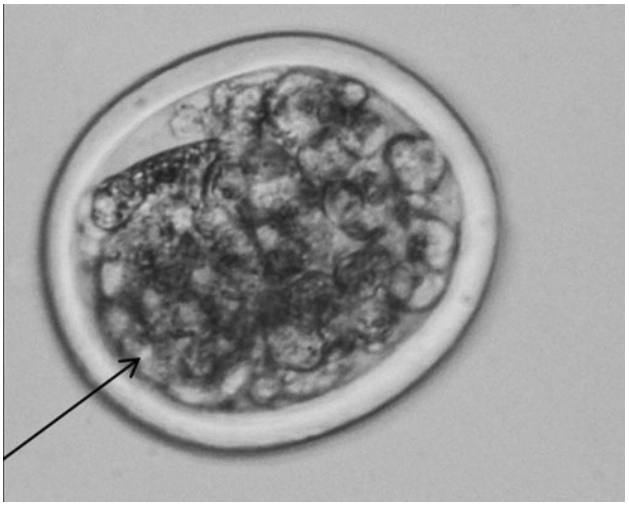


Figure 85.10 Grade 4 morula. Note the small group of cells on the left side (arrow) and very large number of extruded cells on the right. This embryo was identified as grade 4 by our laboratory but may be called grade 3 by others.

is sufficient to cause a lowering of embryo quality grade by one grade (e.g. from quality grade 1 to quality grade 2). Figure 85.12 depicts an early blastocyst-stage bovine embryo with a misshapen zona pellucida.

Lipids in the Cytoplasm

Although lipids are an essential component of cell membranes and must be present in viable embryos, some embryos possess an abnormal accumulation of lipid

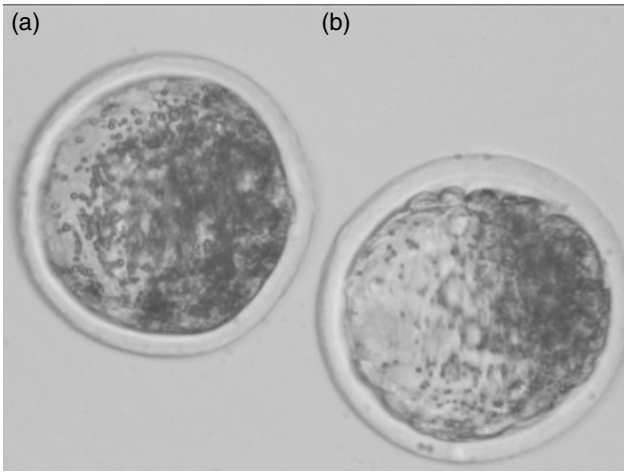


Figure 85.11 Embryos (a) and (b) are both IETS 6–1 embryos. Extruded cells in stages 6 and 7 embryos are often pressed against the zona pellucida and may not be obvious unless the embryo is collapsed. (a) It is difficult to identify this embryo as a blastocyst without rolling the embryo and having a good view of the blastocoele cavity which is obscured by the extruded cells on top. It could be easily mistaken for a degenerating unfertilized ovum.

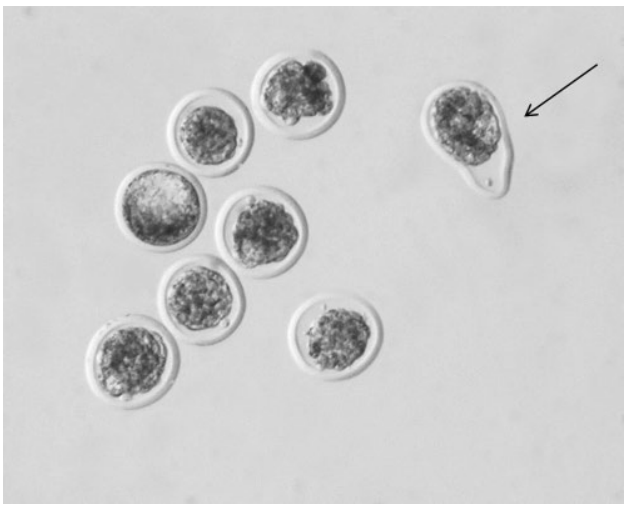


Figure 85.12 The arrow points to a day-7 grade 2 early blastocyst-stage embryo with a misshapen zona pellucida (50×).

droplets (also called vacuoles) within the cytoplasm of individual cells. Most technicians will lower the quality grade of an embryo that possesses more than a few small vacuoles. A high cytoplasmic lipid content may be a cause of decreased pregnancy rates after transfer of frozen–thawed embryos, especially with embryos from the Jersey breed of dairy cattle [22]. Figures 85.13 and 85.14 show the presence of vacuoles within individual cells of bovine embryos.

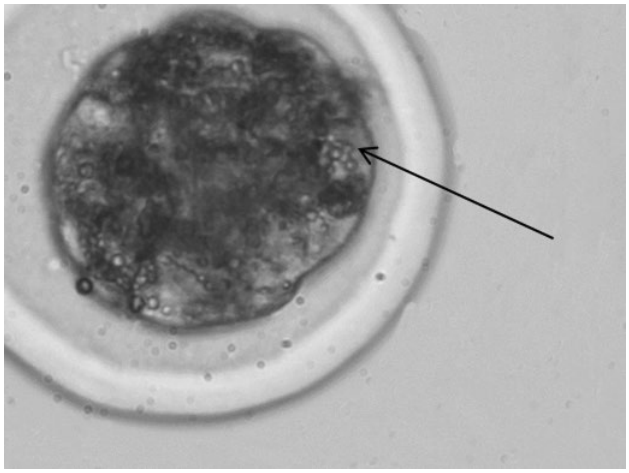


Figure 85.13 Grade 1 compact morula with vacuoles (arrow) in the cytoplasm.

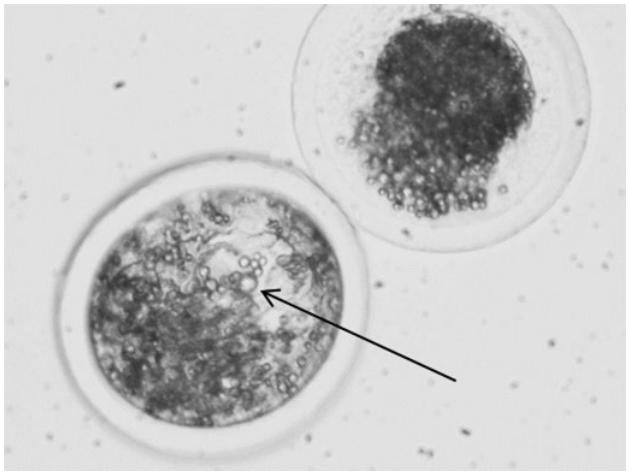


Figure 85.14 Grade 2 bovine blastocyst containing vacuoles (arrow) in the cytoplasm beside an unfertilized ovum. Note that it is difficult to identify the blastocyst embryo without rolling the embryo and having a good view of the blastocoele cavity. Could easily be mistaken for a degenerating unfertilized ovum.

Cracked Zona Pellucida

Typically, a cracked zona pellucida is found only in hatching blastocyst-stage embryos (stage code 8). However, some embryos can exhibit a cracked zona pellucida at earlier developmental stages, presumably due to the hydrostatic pressure placed on the embryo during the embryo recovery process (Figures 85.15–85.17). Although a cracked zona pellucida does not automatically lower the embryo quality grade, only embryos possessing an intact zona pellucida can be washed properly [20] and remain eligible for export. However, they can still be frozen for domestic use.

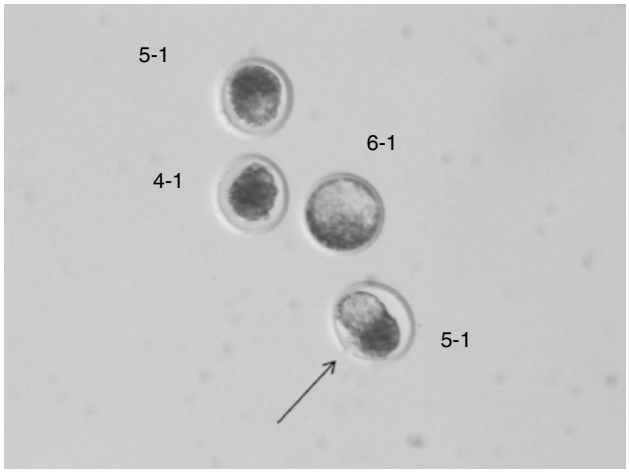


Figure 85.15 Collapsed early blastocyst with a cracked zona pellucida (arrow).

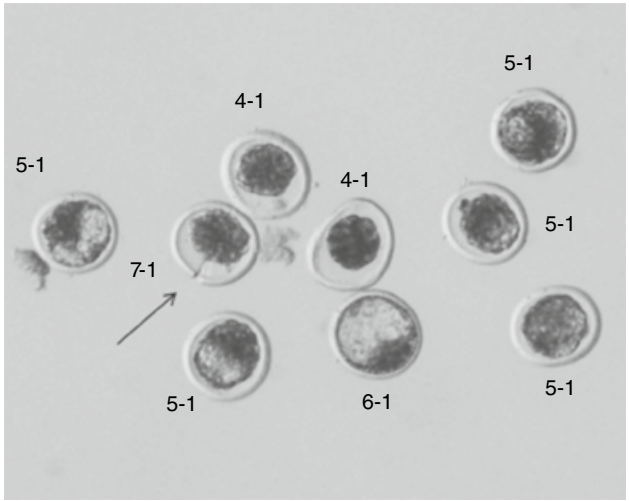


Figure 85.16 Day 7 group of embryos collected from a single donor. Note the embryo with a cracked zona pellucida (arrow) that could be either a morula or a collapsed blastocyst. The thinning of the zona pellucida and slightly larger overall diameter gives supporting evidence that it is a collapsed expanded blastocyst.

Irregular Shape of Cells Comprising the Embryo

An irregular shape of the group of cells comprising the embryo is commonly observed, especially in blastocyst-stage embryos when the blastocoele cavity is forming. Collapsing of the blastocoele cavity is considered a normal physiological process that does not automatically lower the quality grade, except in cases when the zona pellucida is also misshapen. Figure 85.18 depicts a group of embryos with normal and irregular shapes.

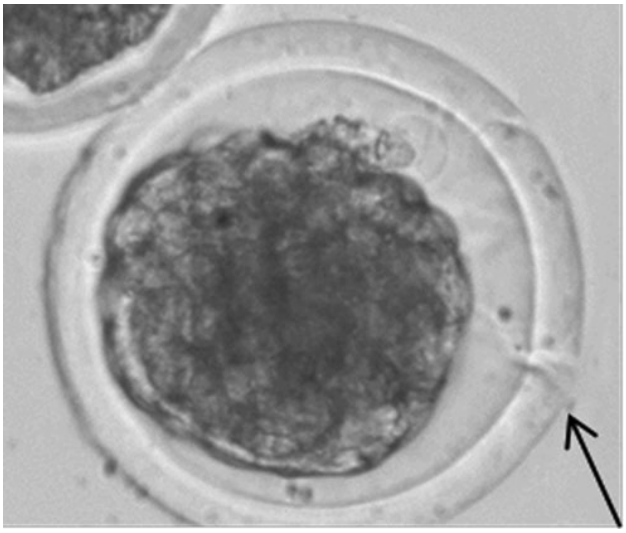


Figure 85.17 Day 7 compact morula. Note the crack in the zona pellucida (arrow).

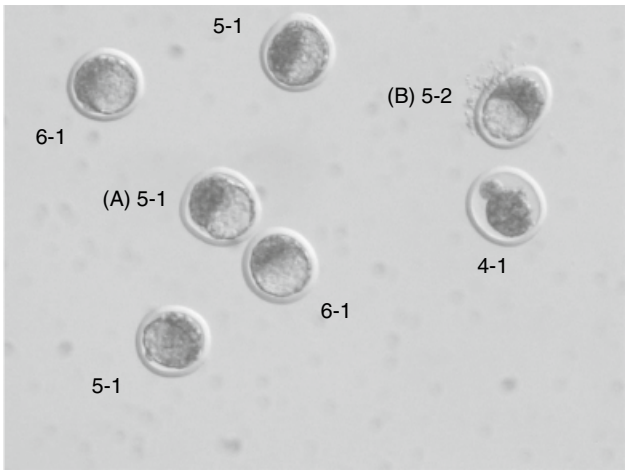


Figure 85.18 Day 7 grade 1 bovine early blastocyst (a) with slightly irregularly shaped cell mass. Day 7 grade 2 early blastocyst (b) with misshapen cell mass and zona pellucida.

Material Adhering to the Zona Pellucida

At the time of embryo recovery, it is not uncommon for some embryos to have material adhered to the outer surface of the zona pellucida. The adherent material may be mucus, individual cells, or a clump of tissue. Adherent material on the zona pellucida may be a sign of endometritis in the donor, cumulus cells that were not removed during normal gamete transport, or donor endometrial cells. Figures 85.19 and 85.20 depict bovine embryos with adherent material. The adherent debris can frequently be washed off the zona pellucida during the IETS-approved embryo washing procedure [20]. In some cases, it may be necessary to include washing in a 0.25% solution of trypsin. Washing embryos with an intact zona pellucida in accordance with IETS washing guidelines should remove the adherent debris, as well as most pathogens.

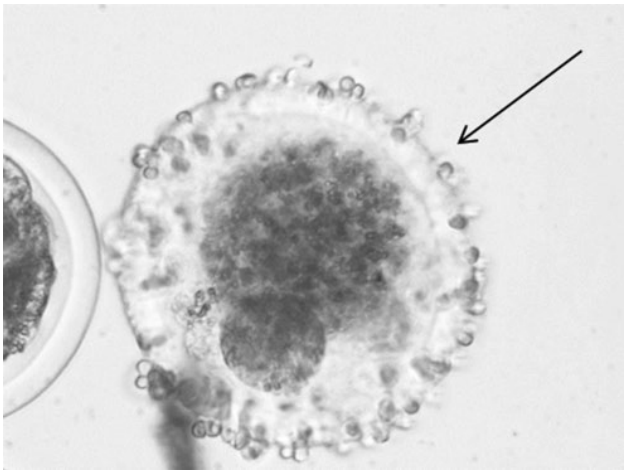


Figure 85.19 Fragmented unfertilized ovum. Note the white blood cells (arrow) attached to the zona pellucida.

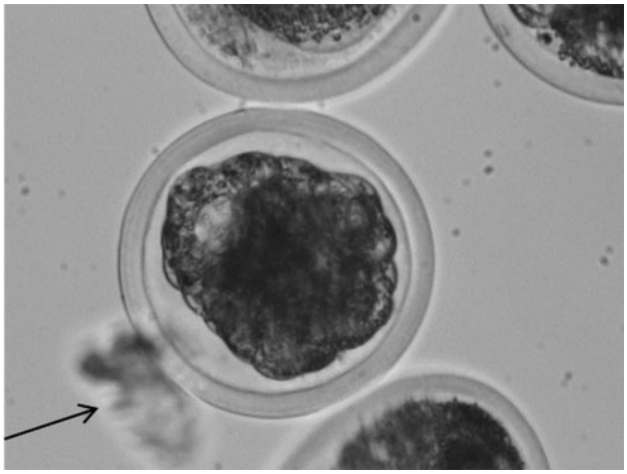


Figure 85.20 Day 7 early blastocyst (5-1). Note the endometrial cells (arrow) adhering to the zona pellucida. These cells must be removed prior to transfer or cryopreservation.

The presence of debris attached to the outer surface of the zona pellucida does not necessarily influence the assignment of an embryo quality grade. However, any embryo with adherent material is not eligible for commercial trade, either domestically or internationally.

Challenges to Accurate Embryo Grading and Classification

One very difficult task for technicians who are learning to grade and classify embryos is to properly distinguish between a compact morula and a UFO, two entities that

can look very similar in size, shape, and color. Figure 85.21 shows a group of compact morula and unfertilized ova side by side, and it is easy to envision how an inexperienced technician may inadvertently describe all these entities as either compact morula or unfertilized ova. This would be especially true when examining the specimens at a lower magnification or with a poor optical quality stereomicroscope. A more highly magnified view of an excellent quality compact morula and a UFO is shown in Figures 85.22 and 85.23. These are good examples of how these two stages of embryonic development are sometimes difficult to differentiate. As stated earlier, it is vitally important to be able to distinguish a compact morula from a UFO to prevent transfer (or cryopreservation) of a UFO and/or the disposal of a compact morula. With sufficient experience in grading embryos, it becomes easier to distinguish these two developmental stages.

Another common misclassification error can occur in instances when a portion of a UFO degenerates and becomes lighter, resembling the blastocoele cavity in a blastocyst-stage embryo (Figure 85.24). Experienced technicians will be able to distinguish the two by looking for the trophoblast layer which "surrounds" the blastocoele cavity in a blastocyst-stage embryo, as well as the ICM cells which are found only in a blastocyst.

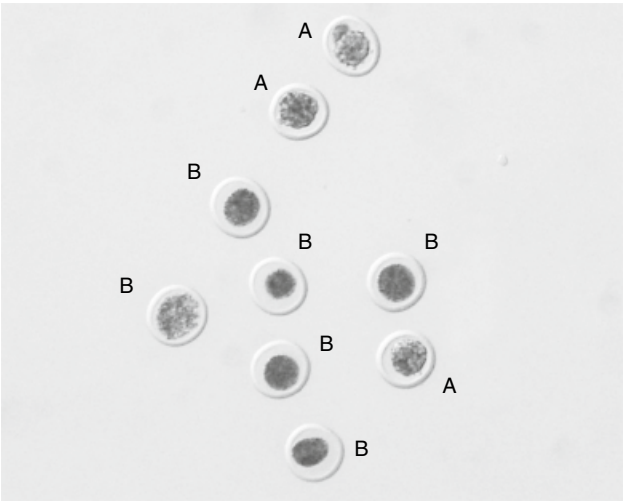


Figure 85.21 Group of compact morula (a) and unfertilized ova (b) recovered from a single donor cow on day 7. Note the similarity in diameter and sphericity between the compact morula and unfertilized ova. Unfertilized ova from this donor appear darker in color, and the vitelline membrane of each ovum is extremely smooth in contrast to the wavy edge of the compact morula.

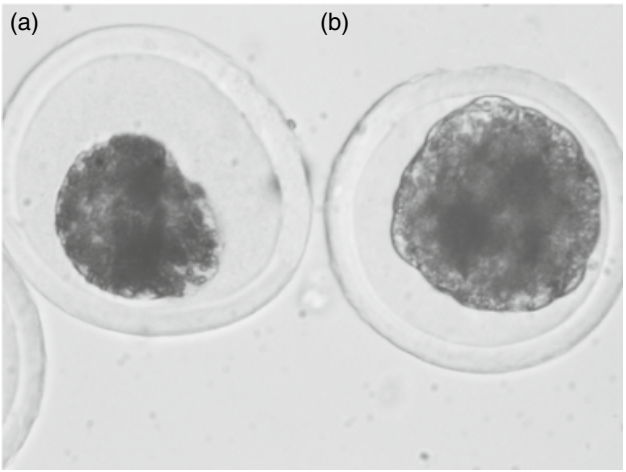


Figure 85.22 An unfertilized ovum (a) and excellent-quality compact morula stage embryo (b).

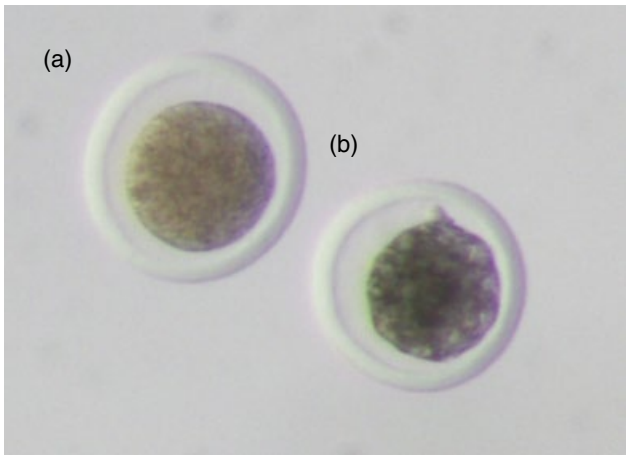


Figure 85.23 An unfertilized ovum (a) and excellent-quality compact morula stage embryo (b).

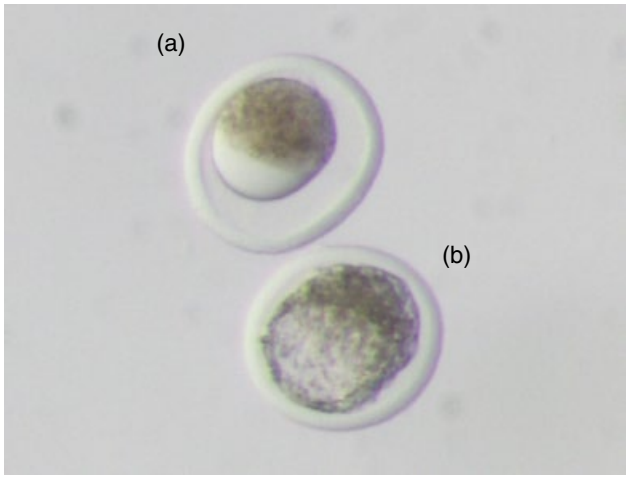


Figure 85.24 A degenerating unfertilized ovum possessing a translucent region (a) and an excellent-quality early blastocyst-stage embryo (b) with discernible trophoblast layer, blastocoele cavity, and inner cell mass cells.

Embryo Evaluation Tip

Although the potential exists for inexperienced technicians to make serious grading and classification errors, experienced embryo specialists seldom make such large mistakes. The most common disagreement among experienced technicians of embryo grading and classification is typically differentiating between excellent/good and fair and between fair and poor quality embryos. This is because of the subjective basis of the embryo evaluation procedure.

To avoid errors in classification and grading of embryos it is vitally important to "roll" the embryos in the Petri dish to enable the technician to thoroughly evaluate the entire surface of the zona pellucida as well as all sides of the cells that make up the embryo. This can be particularly important when donors yield a large number of ova/embryos in a wide range of embryo developmental stages and embryo quality grades (Figure 85.25). A very thorough visual appraisal will reduce the likelihood of embryo grading and classification mistakes.

Each embryo should be examined individually by focusing up and down, keeping in mind that the embryo is a three-dimensional object and that a single two-dimensional view is inadequate for proper evaluation. The embryo should then be rolled (with an embryo handling pipette or by gently and carefully swirling the medium in the dish) to enable the same embryo to be viewed from a different angle. Figure 85.26 consists of three panels (1, 2, and 3) which show the same group of eight embryos from three different angles. Note how different the embryos look in each picture, even though they are the same embryos. These three panels clearly help readers visualize the importance of rolling the embryos around within the Petri dish to enable correct identification of every detail of each embryo. Rolling the embryo and evaluating it from different angles is the key to proper embryo grading and classification.

The Effectiveness of Morphological Embryo Evaluation

Morphological embryo evaluation remains one of the most subjective aspects of the overall embryo transfer process. The photographs presented in this chapter were examined by several embryo specialists who, for the most part, agreed with the stage of development and grade assigned to each embryo. However, there were some mild disagreements over grades assigned to some embryos, and most of the disagreements were between quality grade 1 and 2 embryos. For a couple of the photographs one specialist called a quality grade 1 blastocyst-stage embryo a UFO. These discrepancies most likely occurred from that technician's inability to view the embryos through a stereomicroscope and to roll them around to see them from different angles.

Photographs can be great learning tools, but they are not equivalent to seeing embryos under the stereomicroscope and being able to roll them. One study [23] showed that agreement among six experienced embryo evaluators who viewed video images of embryos was higher for stage of embryo development (89.2%) than it was for embryo quality grade (68.5%). The inability to roll embryos within the Petri dish and to view them from multiple different angles undoubtedly contributed to this lack of agreement. Disagreement among evaluators on embryo stage of development may be attributed to embryos being between two developmental stages. Disagreement on embryo quality

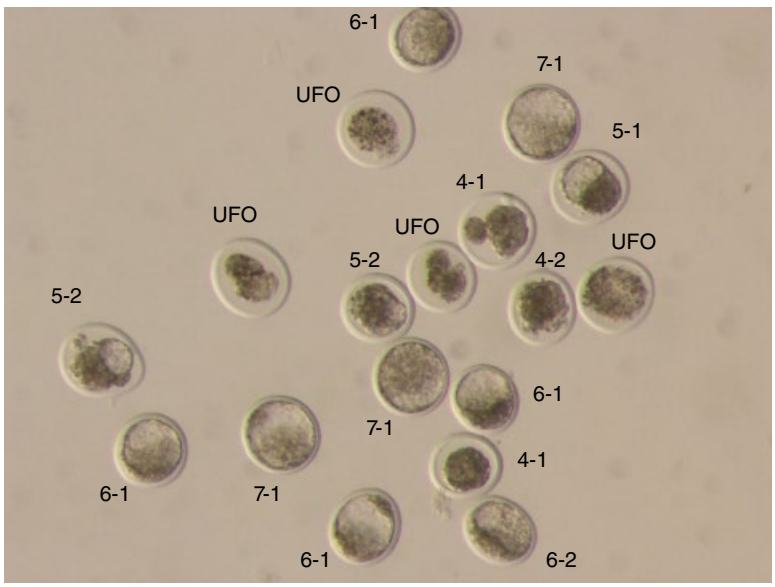


Figure 85.25 A group of 18 day-7 ova and embryos recovered from a single donor.

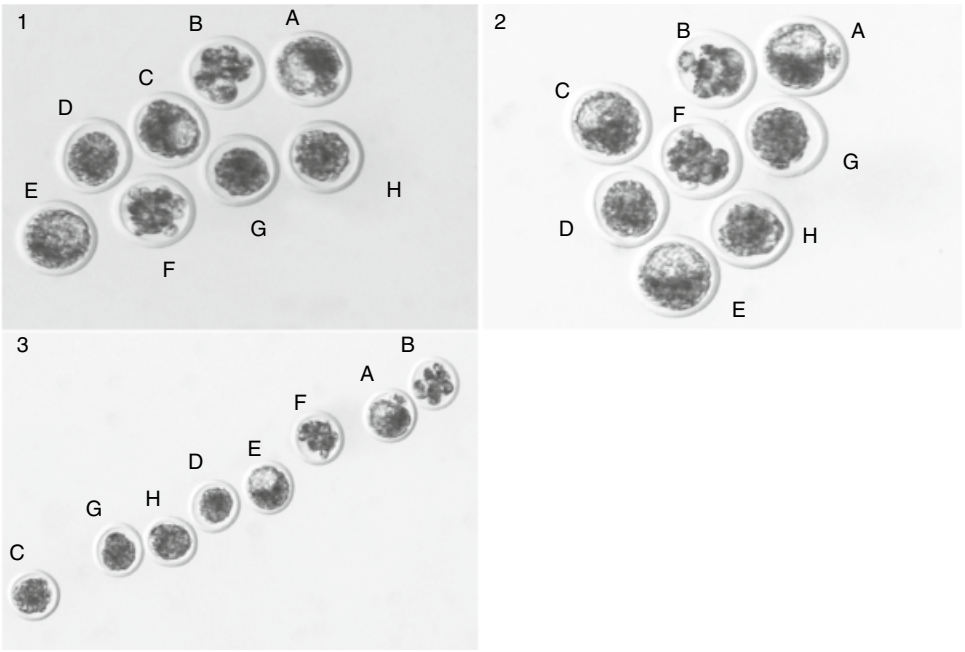


Figure 85.26 A group of eight ova/embryos collected on day 7 from a single donor cow seen from three different angles (panels 1, 2, and 3) after repositioning of embryos within the Petri dish. Note how different each embryo looks in each photo (e.g. embryo C), highlighting the importance of a thorough visual evaluation.

grading was higher when attempting to distinguish between good and fair quality embryos. This likely reflects differences in individual technician philosophies on embryo evaluation. Disagreement was lower when evaluating excellent and degenerate quality embryos. The higher rate of agreement when viewing excellent quality embryos (possessing few, if any, "defects") or degenerate embryos perhaps should not be surprising.

There are three key elements that contribute to successful evaluation of embryos: appropriate training, ample experience, and proper equipment. Appropriate training includes learning the correct morphology of embryos at different times after estrus and the significance of any deviations from normal embryo morphology. One must also learn how to maneuver embryos within a Petri dish in order to thoroughly examine them. Ample experience is gained by examining numerous embryos at all the different stages of embryonic development and different quality grades. Ideally, hundreds of embryos should be evaluated while under the guidance of a highly experienced embryo technician. Experienced personnel can evaluate more than 95% of ova/embryos accurately provided they have a suitable stereomicroscope. Proper equipment includes a good optical quality stereomicroscope of sufficient magnifying capabilities, appropriate embryo handling pipettes (or other handling devices), and satisfactory Petri dishes and embryo media.

Embryo transfer service providers routinely use the IETS embryo grading and classification system. Although this system is subjective, it has allowed practitioners and researchers to record data in a manner that lends itself to subsequent analysis. Although most large embryo transfer datasets originate from the commercial embryo transfer industry, a number of research studies have also contributed to the body of knowledge.

For example, embryonic stage of development has little influence on post-transfer pregnancy rate following transfer of fresh *in vivo* derived embryos to synchronous recipients if the embryos are IETS stage codes 4 (compact morula) to 7 (expanded blastocyst). This result has been obtained in both smaller-scale studies [24] and larger-scale studies involving retrospective analysis of multiple years of data obtained from commercial embryo transfer companies [25]. However, the latter study did document a lower pregnancy rate following transfer of hatched blastocysts (stage code 8).

Similarly, IETS stage code 4, 5, and 6 *in vivo* derived embryos survive the freezing and thawing procedures equally well (i.e. there is no difference in post-transfer pregnancy rate of embryos of the same quality grade). However, embryo quality grade is a critical factor that influences how well embryos survive the freezing and thawing process. Quality grade 1 and 2 embryos typically yield higher pregnancy rates than quality grade 3. One study [24] showed no difference in pregnancy rate among cows receiving grade 1 or 2 embryos (74.1 and 71.8%, respectively), whereas another study [16] showed that

quality grade 1 embryos had a greater probability to conceive (odds ratio 1.62) compared with quality grade 2 or 3 embryos. Conception rate of quality grade 1 embryos was reported as 76% for fresh embryos and 64% for frozen embryos [26].

Conclusion

The ability to accurately evaluate the developmental stage and quality of cattle embryos is critical to the success of an embryo transfer program. Careful examination with a stereomicroscope of ova/embryos recovered from donors is necessary to ensure that viable embryos are not discarded or that unfertilized ova are not transferred or cryopreserved. It is recommended that embryo evaluators undergo intensive and repetitive training and also that a consistent method be used for embryo evaluation. It is highly important to roll the embryos around within the Petri dish to accurately evaluate the entire surface of the zona pellucida as well as all cells comprising the embryo itself. During any given embryo recovery from a superovulated cow one should expect a range of embryo developmental stages and embryo quality grades. The best predictor of an embryo's post-transfer pregnancy rate is its embryo quality grade, with quality grade 1 embryos yielding higher pregnancy rates than either quality grade 2 or 3 embryos. Compact morula (stage code 4) through blastocyst (stage code 6) frozen–thawed embryos are expected to yield equivalent pregnancy rates when transferred to synchronous recipient females. Accurate record-keeping will allow embryo transfer service providers the opportunity to monitor their success and to identify any areas needing potential improvement.

Summary

Embryo transfer is a reproductive biotechnology that facilitates rapid genetic improvement in the dairy and beef cattle industries. An important determinant of the overall success of the embryo transfer process is the quality of embryos obtained from superovulated donor females. Although a variety of different methods have been used over the years to assess the quality of embryos, the only proven and industry-accepted method to assess embryo quality is morphological evaluation. The purpose of this chapter is to provide readers with the necessary information to enable accurate morphological assessment of bovine embryo quality. Although substantial experience is needed before one becomes proficient at this technique, it is an important skill to master because embryo quality is predictive of pregnancy rate obtained after transfer of embryos.

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Control of Embryo-Borne Pathogens

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Introduction

In vivo derived (IVD), *in vitro* produced (IVP), micromanipulated, cloned, and transgenic embryos and oocytes are all vulnerable to contamination from numerous sources, including iatrogenic, the donor, the recipient, semen, culture, and washing media, along with animal source proteins utilized in the media. Bacteria, viruses, protozoans, and prions can result in a variety of negative effects on all types of embryos. Some of these pathogens can be very difficult to detect and may result in just slight decreases in overall embryo production, but others can have significant and often devastating effects on the clinical efficiency of embryo technologies. The epidemiologic adage that the agent, host, and environmental factors will combine to determine whether health or disease will prevail within a population of individuals resounds within the embryo production and transfer industry [1]. It is important to keep this axiom in mind when discussing embryonic pathogens. Three distinct generations of embryo technologies have been developed. With the application of each technology, there have surfaced critical challenges [2]. Each technology implemented imposes environmental changes allowing for the potential to either enhance or diminish the spread of infectious agents among susceptible animals via utilization of these technologies. Hence, it is necessary for these technologies to be extensively examined, as is done yearly by the Health and Safety Advisor Committee of the International Embryo Transfer Society (IETS) [2]. The World Organization of Animal Health (OIE) adopts the committee's recommendations on standards for embryo movement to minimize disease transmission and provide standard protocols and treatments to minimize pathogenic embryonic associations and the validity of experimental treatments currently utilized in embryo technologies to maximize viability and minimize embryonic loss. All areas are important to evaluate when controlling pathogens, but prevention through implementation of biosecurity is the foundation for prevention of all disease states.

The first two generations of embryo technologies, *in vivo* and *in vitro* embryo production, respectively, are widely utilized in commercial cattle production and in basic research. The Data Retrieval Committee of the IETS reported in the December 2018 Newsletter that in 2017 there were approximately 1 487 343 IVD and IVP embryos collected/produced that were available for transfer to other cattle. A total of 495 054 IVD embryos were collected and of these, 162 109 were transferred fresh and 244 178 were transferred frozen. Japan did not report production of IVD embryos in 2017, which most likely accounts for the IVD embryo numbers being decreased by approximately 100 000 [3]. There were 992 289 transferrable IVP embryos produced in 2017 with 757 652 of them transferred. Additionally, there were 500 808 IVP embryos transferred fresh and 256 844 transferred frozen [3]. As far as current numbers for cloned embryos, there were no reported numbers for 2018. There were 105 fresh transfers in 2006 according to the IETS 2006 Newsletter [4]. A number of companies produce cloned embryos, but the current numbers of cloned embryos produced are not accurately known. The USA is currently the largest exporter of embryos, exporting 41.7% of the 26 453 IVD embryos and 65% of the 6133 IVP embryos [3]. The embryo industry is strong and IVP embryo production is increasing every year. It has been estimated that the number of IVD and IVP embryos reported represents about 20% of the embryos produced worldwide [3]. Obviously, there is legitimate concern that due to the level of domestic and international embryo production, that transfer of embryos could facilitate broad distribution of pathogens, resulting in occasional transmission.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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First Generation of Embryo Technologies

In vivo embryo production is considered the first generation of embryo technologies. The original techniques utilized in IVD embryos were developed more than 60 years ago, as detailed in a review by Betteridge [5]. These fundamental procedures are widely used in cattle production today and include superovulation of donors, non-surgical embryo collection, embryo cryopreservation, and non-surgical transfer of embryos to synchronized recipients [6]. The expansive implementation of these reproductive techniques represented a potentially significant environmental change, possibly increasing or decreasing the distribution of indigenous or foreign animal pathogens.

Numerous pathogens of cattle have been evaluated within the context of IVD production and transfer to determine if the techniques might diminish or enhance the normal spread of disease among populations of cattle [7–9]. The original hypotheses were that an intact zona pellucida would protect the early conceptus from infectious agents and that proper handling of zona-intact embryos was likely to reduce and often eliminate rather than enhance the spread of naturally occurring pathogens. There have been four primary strategies utilized to investigate these hypotheses [10]. Initially, zona pellucida-intact (ZP-I) embryos were exposed *in vitro* to a variety of pathogens. These pathogens were chosen due to the regulatory concerns surrounding them, such as institution of and/or current domestic disease control programs and/or international trade restrictions. For example, the OIE considers bluetongue virus (BTV) a reportable disease due to the socioeconomic concern and the major importance in the international trade of animal and animal products [11, 12]. Additionally, venereal, and transplacental transmission exists with most serotypes of BTV, and more pronounced clinical disease can be seen in cattle with serotype 8 [13–17]. Hence, the spread of BTV can be heavily influenced by human interventions that either prevent or facilitate the transmission pathways [18]. This emphasizes the necessity of these trials.

The first strategy consisted of embryos exposed to a pathogen of concern. Following the *in vitro* artificial exposure, embryos were washed with or without trypsin treatment and assayed for the presence of virus, either through implementation of an *in vitro* assay or transfer of embryos to disease-free recipients. The recipients and progeny were monitored closely for evidence of infection. The results of these experiments were promising in that the procedures employed for processing embryos seemed to provide a broad spectrum approach to prevention of spread of several important infectious agents [19, 20]. Additional trials were conducted to validate these embryo processing procedures within the context of normal embryo production and transfer protocols. In these studies, embryos were collected from diseased (acute or convalescent) donors, washed with or without trypsin treatment, and then either assayed for pathogens *in vitro* or transferred to disease-free recipients which, along with their offspring, were monitored for transmission of disease [7–10, 19–21]. The results of these studies served to certify that the embryo processing procedures that are now endorsed by the IETS [21] and recommended by the OIE (in their Terrestrial Animal Health Code, Chapter 4.8.5) [22] are effective widespread deterrents to the spread of certain infectious diseases which are categorized accordingly by the OIE [8, 13, 22, 23]. These procedures include the standard processing procedures for IVD embryos listed in the *IETS Manual* [21] and endorsed by the OIE, again as stated above [22], and the Food and Agriculture Organization (FAO) of the United Nations [24].

The following procedures are recommended:

1) Embryos should be washed at least 10 times with at least 100-fold dilutions between each wash, and a fresh pipette should be used for transferring the embryos through each wash.
2) Only embryos from the same donor should be washed together, and no more than 10 embryos should be washed at any one time.
3) Sometimes, for example when inactivation or removal of certain viruses, such as bovine herpesvirus-1 and Aujeszky's disease virus, is required, the standard washing procedure should be modified to include additional washes with the enzyme trypsin, as described in the *IETS Manual* [21].
4) The zona pellucida of each embryo, after washing, should be examined over its entire surface area at not less than 50× magnification to ensure that it is intact and free of adherent material [21, 24].

The protocol for washing and trypsin treatment for adherent viruses such as infectious bovine rhinotracheitis virus (bovine herpes virus-1), Aujeszky's disease, and vesicular stomatitis requires that the embryos be washed five times in phosphate-buffered saline to which antibiotics and 0.4% bovine serum albumin have been added, but without Ca^{2+} and Mg^{2+} (because they inhibit trypsin) [21]. Next the embryos should be put through two washes of trypsin of 60–90 seconds each, then again through five washes of saline containing antibiotics and 2% heat-inactivated serum (not bovine serum albumin) to inactivate the trypsin [21, 24]. The trypsin enzyme used to prepare the washes should have an activity such that 1 g will hydrolyze 250 g of casein in 10 minutes at 25 °C and pH 7.6 [21]. The sterile trypsin solution should contain a concentration of

0.25% trypsin in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} [21]. There are ready-to-use solutions commercially available. After embryos have been washed, they should be microscopically examined over their entire surface area at no less than 50× magnification [21, 24]. Also, for purposes of disease control, only embryos that have an intact zona pellucida and that are free of adherent material should be transferred or cryopreserved [21–24].

Sources of Pathogens

Some areas of embryo production have seemed to receive adequate attention, while other important sources of pathogens in embryo production have received insufficient attention. These sources of pathogens are contaminated materials (reagents) of animal origin and diseased donors and recipients [6, 25, 26]. The term "materials of animal origin" embraces a variety of things, including supplements to media (e.g. fetal bovine serum [FBS] or bovine serum albumin), hormones that might be used to treat donors (e.g. pituitary-origin follicle stimulating hormone), and enzymes for treatment of embryos (e.g. trypsin). Detailed descriptions of the hazards associated with these products have been previously described [25–27]. To highlight some, bovine serum albumin is an unlikely source of pathogens because production protocols seem to preclude the survival of contaminants [28, 29]. However, on the other hand, FBS could be the source of adventitious bacteria, fungi, or viruses when adequate quality assurances are not in place [25, 30]. The most common contaminant in FBS is bovine viral diarrhea virus (BVDV) [25, 30–32].

A number of pathogens are known to be transmitted from mother to fetus via the placenta. These include BVDV, *Neospora caninum*, BTV, bovine leukemia virus (BLV), bovine herpesvirus (BHV)-1, and *Mycobacterium avium* ssp. *paratuberculosis* (Johne's disease) [13–17, 33–35]. Thus, a complete risk assessment should always give due consideration to the entirety of infectious agents capable of establishing transient, latent, or persistent infection of the donor and/or recipient, which can result in fetal disease. Often the disease may spread from the recipient to other animals through horizontal transmission. Otherwise, collection and transfer of embryos, albeit even pathogen-free embryos, could lead to the birth of diseased offspring, resulting from subsequent in utero infections.

As of 2019, the official biosecurity recommendations for international movement of embryos are outlined in Section 4.8.5 of the Terrestrial Animal Health Code under "Risk Management" [36]. These general strategies were developed to help ensure that the transfer of IVD embryos would not result in the transmission of infectious agents originating from donor animals [36]. Three general strategies are available [37, 38]. Each method represents a contrived environmental change intended to deter pathogen transmission. The preferred method in cattle is embryo processing (i.e. washing or trypsin treatment) [21] (as previously described in the section "First Generation of Embryo Technologies"), because it is relatively inexpensive and has been substantiated by a considerable amount of research to prevent the spread of a variety of viruses, bacteria, and bovine spongiform encephalopathy [8, 22, 36–38].

Other methods have been employed in cattle, which include donor testing and recipient quarantine and testing [37]. By testing donors for the presence of disease at the time of embryo collection, and again, weeks or months later (while embryos are held in the cryopreserved state), it can be confirmed that the donors are not a possible pathogen source. The testing of recipients following transfer of embryos and testing of offspring after birth function as bioassays utilizing the recipients and offspring as sentinel animals. However, this requires a lengthy quarantine of animals. Additional disadvantages are that these methods are expensive, multiple tests have to be conducted if there is a need to detect more than one disease, and multiple testing must be done to determine that accidental exposure did not occur during the quarantine period. Therefore, economically, the use of embryo processing is the preferred method for certifying the health of embryos. This method has greatly facilitated international trade in embryos because of the combination of animal welfare, disease control, and economic advantages [37].

Categorization of Pathogenic Agents of Embryos

In order to further facilitate international trade of embryos, an estimation, or categorization, of the known risk of transmission of a particular disease via embryo transfer (ET) is compiled by the OIE through the help of the Health and Safety Research Subcommittee of the IETS and is updated yearly [22, 23, 36]. The official categorization of diseases of embryos from one to four is detailed further in Article 4.8.14 which can be found by accessing Article 4.8.4 "Conditions applicable to the introduction of donor animals" in Section 4.8 of the Terrestrial Animal Health Code [22]. Categorization is currently under review by the IETS's Health and Safety Advisory Committee (HASAC) Research Subcommittee and some changes may occur in the coming years. As for now, Category 1 diseases are diseases or pathogenic agents for which sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled

between collection and transfer in accordance with the *IETS Manual* [21–24, 36]. The following diseases or pathogenic agents are in Category 1: BTV (cattle), bovine spongiform encephalopathy (cattle), *Brucella abortus* (cattle), enzootic bovine leukosis (cattle), foot and mouth disease (cattle), infection with Aujeszky's disease virus (pigs): trypsin treatment required, infectious bovine rhinotracheitis/infectious pustular vulvovaginitis: trypsin treatment required, and scrapie (sheep) [22].

The Terrestrial Animal Health Code 4.8.14 states that Category 2 diseases are those for which substantial evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the *IETS Manual* but for which additional transfers are required to verify existing data [22]. The following diseases are in Category 2: bluetongue (sheep), caprine arthritis/encephalitis, and classical swine fever. Category 3 diseases or pathogenic agents are those for which preliminary evidence indicates that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the *IETS Manual* but for which additional *in vitro* and *in vivo* experimental data are required to substantiate the preliminary findings [22]. The following diseases or pathogenic agents are in Category 3: atypical scrapie, bovine immunodeficiency virus, bovine spongiform encephalopathy (goats), BVDV (cattle), *Campylobacter fetus* (sheep), foot and mouth disease (pigs, sheep, and goats), *Haemophilussomnus* (cattle), maedivisna (sheep), *Mycobacterium paratuberculosis* (cattle), *N. caninum* (cattle), ovine pulmonary adenomatosis, porcine reproductive and respiratory disease syndrome, rinderpest (cattle – eradicated), porcine circovirus type 2 (pigs), and swine vesicular disease [22]. Category 4 diseases and pathogenic agents as listed by the OIE in the Terrestrial Animal Health Code 4.8.14 and are those for which studies have been done, or are in progress, that indicate no conclusions are yet possible with regard to the level of transmission risk; or that the risk of transmission via ET might not be negligible even if the embryos are properly handled according to the *IETS Manual* between collection and transfer [22]. The following diseases or pathogenic agents are in Category 4: African swine fever, akabane (cattle), bovine anaplasmosis, BTV (goats), border disease (sheep), bovine herpesvirus 4, *Chlamydia psittaci* (cattle, sheep), contagious equine metritis, enterovirus (cattle, pigs), equine herpes virus 1 (equine rhinopneumonitis), equine viral arteritis, *Escherichia coli* 09:K99 (cattle), *Leptospira borgpetersenii* serovar *hardjobovis* (cattle), *Leptospira* sp. (pigs), lumpy skin disease, *Mycobacterium bovis* (cattle), *Mycoplasma* spp. (pigs), ovine epididymitis (*Brucella ovis*), parainfluenza-3 virus (cattle), parvovirus (pigs), Q fever (*Coxiella burnetii*), scrapie (goats), *Tritrichomonas foetus* (cattle), *Ureaplasma* and *Mycoplasma* spp. (cattle, goats), and vesicular stomatitis (cattle, pigs) [22].

Control of Viral Pathogens

Considerations of space preclude discussion of each disease listed in all four categories. Therefore the primary focus of the remainder of this chapter will be on BVDV, which is of great importance due to its tremendous economic impact on the cattle industry as a whole. This pestivirus, BVDV, is a Category 3 disease. It has the ability to diminish production through decreases in feed efficiency and milk production, and to cause early embryonic death, abortion, mummification, fetal abnormalities, diarrhea, pneumonia, and other various debilitating disease states [27, 39–46]. The economic impact of BVDV was evaluated in a study by Houe, who estimated BVDV losses to the cattle industry to be between USD $20 and 57 million per million calvings [40]. Hessman et al. investigated the economic effects and the health and performance of cattle populations after exposure to persistently infected (PI) animals [41]. They reported that for cattle with direct exposure to a PI animal, economic losses due to fatalities were $5.26 per animal and performance losses were much more significant at $88.26 per animal [41]. Also, due to the significant economic implications resulting from BVDV infections, a number of countries such as Denmark, Finland, Norway, Sweden, the Shetland Islands, and Slovenia have established eradication programs [27, 47–51]. Several countries are utilizing control programs and are moving toward implementation of eradication programs, while others, such as the USA, are leaving the individual states and specifically the producers within those states to set standards for BVDV control by the formation of voluntary control programs. Control of BVDV in the USA is further complicated by the existence of BVDV in the white tail deer population [52, 53]. Hence, the focus of most control programs is appropriate biosecurity including quarantine, testing, identification, and removal of PIs and prevention of PI formation.

Embryo Transfer as a Means of Controlling Pathogen Transmission

Historically, ET was utilized as a way to minimize disease transmission along with maximizing genetics [20, 54]. However, in the years following its inception, questions regarding the ability of embryos to transmit pathogens

surfaced. Numerous studies were performed that showed that ET was indeed a good way to prevent or minimize pathogen transmission if good quality controls and appropriate processing procedures were instituted [8, 20, 37, 38, 54–59]. However, there were some organisms for which prevention of pathogen transmission was not refutable and in some cases ET might plausibly serve as a mechanism for introduction of disease. The original placement of BVDV in Category 3 was based on a study conducted in 1982 [54]. This study validated the efficacy of embryo washing procedures for removal of virus after artificial exposure of embryos to a cytopathic isolate of BVDV [54]. Four later studies collectively produced five BVDV-free calves after transfer of washed or trypsin-treated embryos from PI donor cows [55–58]. While data from these four reports were limited, the results were encouraging. However, reports by Lindberg et al. and Drew et al. [60, 61] stimulated further investigations into the possibility of transmission of BVDV via ET due to seroconversion of heifers after ET and birth of a PI calf following ET, respectively. Contaminated FBS was thought to be the inciting cause of these infections. Hence, additional studies were necessary to determine if BVDV would remain associated with BVDV following standard IETS processing procedures for IVD embryos and whether this associated virus could be transmitted via ET.

So, further studies were performed which highlighted the variation in affinity between different strains of BVDV and the affinity of these strains for embryos [62, 63]. Some strains maintained association with embryos following IETS processing procedures, including when trypsin treatment was added to the processing procedures [62, 63]. These high-affinity strains became more of a concern when it was determined that the embryo-associated virus (EAV) was indeed infective in both *in vitro* and *in vivo* studies [63, 64]. Gard et al. (2009) [65] reported that 27% of IVD and 42% of IVP embryos had EAV following artificial exposure to a high-affinity strain of BVDV (SD-1, type 1a) following washing procedures in accordance with IETS (without trypsin). The range of the amount of EAV was 100–450 cell culture infective doses (CCID) to the 50% endpoint ($CCID_{50}$)/embryo [65]. In previous studies, EAV was also determined to be infectious in an *in vitro* culture (IVC) system and in an *in vivo* model through intravenous inoculation of embryos into virus negative and seronegative recipients [62, 64, 65]. An additional study was performed that utilized twice the amount of EAV known to be previously associated with individual IVD and IVP embryos following processing procedures [66]. This amount was then doubled (878 $CCID_{50}$/straw) to imitate what is commonly done in the ET industry, transferring two embryos simultaneously into one recipient, and in keeping with the worse-case scenario. It was found that all recipients of embryos and virus became viremic, and then seroconverted. There were six pregnancies at 30 days and only one at 60 days post transfer. However, all pregnancies were lost. The 60-day pregnancy was removed via colpotomy and tested for BVDV. The aborted fetus was found to have the same strain of BVDV, SD-1, as the infecting viral strain [66].

The finding that no BVDV-positive live offspring were produced in this study is similar to the results of the study by Bielanski et al. [67]. In this study, IVP embryos were exposed to non-cytopathic biotypes of BVDV for one hour, either type 2 strain (P-131) or type 1 strain (NY-1), and then washed in accordance with IETS guidelines (no trypsin treatment) then transferred to seronegative and BVDV-negative recipients. However, none of the recipients following intrauterine transfer of embryos exposed to the type 1 strain seroconverted, but of the 35 recipients receiving embryos exposed to the type 2 strain, 18 seroconverted (51%) and there were 11 pregnancies at 30 days post transfer. Of the 11 pregnancies only two resulted in live offspring. These two offspring were tested and determined to be BVDV seronegative and negative for antigen [67]. The infection may not have been recognized by the fetus and/or the virus was destroyed prior to development of immune competence so in fact no antibodies were formed by the fetus. The results of no detectable seroconversion from the type 1 strain and abortion due to the type 2 BVDV may be the results of mutations within the virus. In a study by Meyers et al., decreases in interferon production, abortion, and presence of virus in fetal tissues did not result when pregnant cattle were injected with two different mutant strains of virus [68]. Each of these mutant strains had mutations specifically affecting both the N-terminal protease, N(pro), and the deletion of codon 349, which abrogates the RNAse activity of the structural glycoprotein E(rns) [69]. However, decreases in interferon production, abortion, and presence of virus in fetal tissues did occur with wild-type viruses and in viruses in which only one mutation of either N(pro) or E(rns) occurred [68]. Therefore the establishment of persistent infections requires both N(pro) and E(rns). It is logical to ascertain that the type 1 strain utilized in Bielanski's study might have had mutations within these areas resulting in adequate interferon production and no fetal infection. Each specific strain may affect interferon production differently and therefore may or may not result in fetal infection and that fetal infection may or may not be fatal.

Thus, based on these studies and the variety of BVDV strains, it is still plausible that a PI could result via transfer of a contaminated embryo. The results from Gard's and Bielanski's studies show that some high-affinity isolates can result in viremia and seroconversion in susceptible

recipients followed by early embryonic death and abortion [66, 67]. The possibility of the development of a PI following transfer of contaminated embryos seems to be more likely to result from the viremia of the recipient (acute exposure) and shedding of virus to other gestating animals than from transfer of a contaminated embryo, based on the findings of these studies. However, additional studies involving naturally infected embryos with various strains of BVDV transferred intrauterine need to be completed in order to establish a thorough investigation of the ability of BVDV to be transmitted intrauterine and the potential for development of a PI calf. A thorough evaluation is important as the ability to transmit BVDV via an embryo would necessitate reevaluation of embryo health certification procedures along with implementation of additional regulations on embryos exported from BVDV-positive countries to those countries where BVDV has been eradicated.

Second Generation of Embryo Technologies

In vitro embryo production has been described as the second generation of embryo technologies and most of the essential components of this process in cattle have been developed and refined as these procedures have become adapted for commercial use [27, 38, 70]. Fundamental procedures currently used with *in vitro* production include superstimulation/superovulation of donor cattle, collection of oocytes via transvaginal follicular aspiration, *in vitro* maturation of oocytes (IVM), *in vitro* capacitation of spermatozoa, *in vitro* fertilization (IVF), IVC of embryos to blastocysts, and ET to a recipient [71]. This technology represents multiple changes in the animal breeding environment which provides opportunities for pathogens to enter, amplify, and be disseminated. Hence, there are a number of logical sources of pathogens in IVP embryo production. These sources include gametes and their associated fluids, somatic cells used in IVM, IVF, or IVC, and the materials of animal origin used to supplement maturation, fertilization, or culture media (e.g. FBS, bovine serum albumin, or follicle stimulating hormone [FSH]) [25].

Studies that surveyed for contaminants in *in vitro* embryo production laboratories have been conducted in a number of different countries such as Canada [69, 72], Denmark [73], France [7, 74], and the USA [70, 75, 76]. As expected, levels of bacterial contamination can be quite high – from 13 to 68% of samples when abattoir-origin materials are utilized in *in vitro* production systems [70]. Also, low to moderate levels of BVDV and BHV-1 have been found in abattoir-origin materials, with a range of one to 12% of samples testing positive for BVDV and zero to 12% of samples testing positive for BHV-1 [70, 76]. Luckily, the majority of IVP embryo production occurs without utilizing abattoir origin oocytes or uterine tubal cells. According to the Data Retrieval Committee of the IETS in 2017, only 1.2% of IVP embryos transferred were from abattoir-origin oocytes [3]. Additionally, in 2017, no IVD or IVP exported embryo was produced utilizing abattoir-origin oocytes [3]. However, there are other materials from the abattoir that are utilized in reproduction in cattle on a wide basis such as fetal calf serum and follicle stimulating hormone (FSH) that still have potential for contamination.

Since BVDV is widely distributed among populations of cattle and is known to result in severe respiratory, gastrointestinal, and reproductive disease, it has received the most attention by researchers [75]. In fact, all of the materials (animal products, ovaries, oocytes, follicular fluid, cumulus, uterine tubal cells, FSH, and serum) of animal origin that have been integral to IVF embryo production in cattle have been shown to contain BVDV when harvested from actively infected animals [75, 77]. This hazard is complicated by the fact that a proportion of animals infected by BVDV are asymptomatic [32, 40]. The PI cattle are often asymptomatic and, as stated, they serve as reservoirs for large quantities of virus [40]. Also, most of the field isolates of BVDV are noncytopathic and could cause unapparent persistent infections in donor animals from which semen, oocytes, and embryos are collected and from which laboratory cell lines are established and that are in co-cultures with oocytes and embryos [30, 77]. Finally, it is noteworthy that the cultures in each phase of IVF embryo production are ideally suited for replication of BVDV, providing adequate time and substrate as well as permissive cells for viral replication, such as the cumulus cells and spermatozoa. Therefore even a low level of contamination can be effectively amplified, leading to exposure of embryos to a high level of virus by the end of IVC. However, synthetic oviductal media have been developed to minimize contamination through use of animal origin products, but cumulus cells, semen, oocytes, and embryos will always provide a route for contamination in culture systems.

As with IVD embryos, it was originally hypothesized that the zona pellucida of IVF embryos would protect the developing conceptus from infections and, in fact, artificial exposure studies have demonstrated that an intact zona is an effective barrier that is seldom penetrated [78]. Nevertheless, it is clear that the processing procedures recommended for use on IVD embryos are not as effective for IVF embryos, since several pathogens such as BTV, BHV-1, foot-and-mouth disease virus, and BVDV have been shown to adhere more readily to the zona of IVF embryos [75, 76]. Furthermore, the presence of virus has been shown to result in reduced rates of maturation, fertilization, and development [75, 79–82].

Studies have shown that BVDV remains associated with IVP embryos after viral exposure and washing with or without trypsin treatment [67, 83, 84]. Infection of susceptible recipients was reported in Bielanski's study where 18 of 35 naïve recipients receiving embryos exposed to the BVDV type 2 strain seroconverted (51%) and only two of 11 pregnancies resulted in live offspring. These two offspring were determined to be negative for BVDV, which leads to the conclusion that the embryos with EAV underwent embryonic death [67]. Additionally, some interesting results were achieved in attempts to determine whether the amount of BVDV associated with individual embryos would constitute an in-utero, infective dose [85, 86]. The cumulative results of these *in vitro* studies evaluating risks of transmitting BVDV via ET of IVP embryos are somewhat contradictory. On the one hand, it is clear that multiple strains of BVDV will remain associated with IVF embryos after washing or trypsin treatment [85–87]. On the other hand, there appear to be innate deterrents to transmission of the EAV to permissive cells *in vitro* which mimics the environment of the uterus and uterine tube [85–87]. Additionally, media free of animal origin products are being produced and utilized that decrease the chances of entry of BVDV into the culture system, but one cannot ignore that there can be EAV. Obviously, resolution of these issues requires further studies.

Official recommendations for international shipment of IVP embryos in order to control the spread of disease are outlined in Section 4.9 of the Terrestrial Animal Health Code of the OIE [88]. Impediments to the establishment of universally recognized sanitary precautions for the production of bovine IVF embryos include lab-to-lab variability in techniques utilized, the comparative ease with which pathogens adhere to the zona pellucida, and the fact that raw materials, such as FBS, are collected from abattoirs [89]. It is apparent that mechanical washing and trypsin treatment of IVF embryos [83, 84] do not provide the same level of effectiveness when utilized for cleansing IVD embryos. However, these treatments have been shown to definitely reduce the level of contamination after IVF embryos following artificial exposure to some agents such as BHV-1 and BVDV [83, 84, 90]. Thus, as is the case with IVD embryos, further studies are necessary in order to complete the risk assessment which includes evaluating fully if the quantity of embryo-associated BVDV is sufficient to infect recipients and/or their fetuses after transfer into the uterus. Obviously, the strain of the virus seems to have a tremendous effect on the affinity of the virus for embryos and it is necessary to take this into account when assessing the true risk [63].

Third Generation of Embryo Technologies

The third generation of embryo technologies includes cloning, such as somatic cell nuclear transfer, and transgenics. These technologies specifically involve maturation and enucleation of oocytes, insertion of somatic cell nuclei, activation through chemical and/or electrical means, IVC, possibly cryopreservation, and non-surgical transfer. The same hazards that are associated with the second generation of embryo technologies apply directly to the third generation of embryo technologies with a few added caveats. These caveats include that the zona pellucida is either broken or removed, and the donor nuclei are harvested from additional cell lines. These cell lines utilized are often cultured for 8–16 weeks in order to allow for cellular amplification, and even longer if a transgene has been inserted. Additionally, the amplification procedures can allow for non-cytopathic BVDV replication and infection in these cell lines due to the use of animal origin materials such as abattoir origin oocytes and FBS utilized in formation and culturing of embryos. Logically, cloning has a much greater chance for contamination than IVD and IVP production.

The presence of non-cytopathic BVDV may be manifested in poor cloning efficiency and development, low pregnancy rates, early embryonic death, fetal wastage, and neonatal abnormalities. An example of the problems that might be associated with BVDV contamination in cloning systems is well documented in a report by Shin et al. 2000 [91].In this report, Shin described a high incidence of developmental failure in bovine fetuses that were derived by cloning using BVDV-infected cells. Specifically, a fetal fibroblast cell line was inadvertently infected with a non-cytopathic strain of BVDV. As a result of the infection an extraordinary amount of embryonic death and fetal resorption occurred with the use of this cell line [91]. In an additional study by Stringfellow et al. [92], screened samples sent to veterinary diagnostic labs revealed that 15 of 39 fetal fibroblast cell lines used in cloning research were positive for BVDV as determined by various assays including reverse transcription-polymerase chain reaction (RT-PCR). However, it was determined that only five of the 39 cell lines were actually infected with BVDV. Furthermore, three of these five lines were determined to not be infected at the earliest cryopreserved passage – hence leading to the conclusion that the cell lines became infected after culture in media containing contaminated FBS. Sequence comparison of the amplified cDNA from one lot of FBS confirmed that it was the source of infection for one of these cell lines [92]. Since BVDV was isolated from the remaining two cell lines at the earliest available passage, the fetuses

from which they were established could not be ruled out as the source of the virus. These studies highlight the additional concerns that cloning and transgenics pose and the greater potential for the production of contaminated embryos via the utilization of these techniques. Therefore excellent quality control is imperative in order to prevent embryonic pathogens and transmission of pathogens via ET to recipients. It is of major importance with all generations of embryo technologies and the needs increase with each subsequent generation.

The use of embryo technologies continues to increase in other species including swine, caprine, ovine, and equine, and wildlife as well. In 2017, 18 652 sheep, 3076 goat, and 20 459 horse IVD embryos were transferred. Additionally, there was an increase in equine IVP embryos with 14 788 embryos transferred [3]. Intracytoplasmic sperm injection (ICSI) continues to grow in the equine and bovine industries as well. During ICSI, the zona pellucida is punctured and a single spermatozoa is injected into the oocyte. Then the embryo is cultured in a similar fashion as an IVP embryo and finally the embryo is transferred. Good quality control and biosecurity are a must to reduce the chances of embryo and fetal mortality when using ICSI. Appropriate processing of oocytes and spermatozoa are crucial. Again, donors and recipients can be vectors of disease, and as is the case in cattle, there are a variety of pathogens that pose a concern and can result in embryonic and fetal mortality. A review by Givens and Marley [93] summarizes bacterial, fungal, protozoan, and viral causes of reproductive dysgenesis in cattle, sheep, goats, pigs, horses, dogs, and cats. This study illustrates how it is imperative to make prudent use of immunization and biosecurity protocols when incorporating embryonic technologies in order to minimize reproductive losses.

Experimental Treatments

The addition of antibiotics and antifungals to media significantly decreases problems with bacteria and fungi in culture. However, viral contamination poses a more complicated threat. A study looked at the addition of an antiviral substance, interferon-tau, to embryos in culture with BVDV and BHV-1. A significant decrease in the amount of BVDV in culture was seen but the same result was not observed for BHV-1 [94]. Additionally, other antiviral compounds such as DB606 (2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan) have been shown to inhibit the replication of BVDV in bovine uterine tubal epithelial cells, Madin Darby bovine kidney cells, and fetal fibroblast cells, without negatively affecting embryonic development or future fertility of heifers derived from IVF embryos treated with DB606 [95, 96]. A study by Newcomer et al. [97] evaluated the scope of antiviral activity of another similar compound, 2-(2-benzimidazolyl)-5-[4-(2-imidazolino) phenyl] furan dihydrochloride (DB772), among diverse pestiviruses. Isolates of BVDV-2, border disease virus (BDV), HoBi virus, pronghorn virus, and Bungowannah virus were all tested for *in vitro* susceptibility to DB772 by incubating infected cells in medium containing various concentrations of DB772. It was found that DB772 effectively inhibits all pestiviruses studied at concentrations >0.20 μM with no evidence of cytotoxicity [97]. These compounds, DB606 and DB772, along with interferon-tau, show potential for minimization of viral pathogens, specifically pestivirus, in culture, which may result in their routine inclusion during culture of embryos and amplification of fibroblast cell lines for cloning and transgenics [96–98].

Emerging pathogens such as HoBi-like viruses (HoBiPeVs), which have also been called "atypical pestiviruses" and "BVDV-3," are of special concern since they cannot always be detected via testing for BVDV and BVDV vaccines may have limited efficacy against these viruses. These HoBiPeVs are found in multiple countries from Brazil to Asia and Europe. The use of contaminated FBS is implicated as the source through which HoBiPeVs have moved worldwide [99]. In Brazil, HoBiPeVs are endemic and account for 19.9% of the genotyped pestiviruses in 2018 [100]. Brazil has the biggest cattle herd and is still the largest producer of IVP embryos [3, 100]. Hence, HoBiPeV positive donor cattle can be seen as a significant source of contamination as well. Hence, efficacious antivirals would be beneficial in preventing EAV and possible transmission of HoBiPeVs since not much research has been completed involving bovine embryos and HoBiPeVs.

Human hepatitis C virus (HCV) is very similar to bovine pestiviruses. Pegylated interferon (PEG-IFN) and ribavirin have been considered the standard of care for HCV in humans but only have a sustained virologic response rate (SVR) and lack production of HCV RNA in 40–80% of patients depending on HCV genotype [101]. New antiviral drugs called direct-acting antivirals (DAAs) are now on the market and have SVRs as high as 60–100%. These DAAs target the non-structural HCV proteases NS3-4A and NSA5 or inhibit RNA-dependent RNA polymerase, and so they are termed protease inhibitors and inhibitors of HCV RNA-dependent RNA polymerase, respectively, which could be efficacious against HoBiPeVs [101]. Zika virus is another emerging disease that has looked to antivirals to inhibit the RNA-dependent

RNA polymerase to prevent severe repercussions of the virus on the embryo and fetus [102]. Zika virus is in the same family as BVDV and HCV, Flavivirdae [39, 40, 102]. Obviously, these new drugs regimens can have applications in human and bovine medicine. The use of antivirals would be extremely beneficial to counteract infectious virus on embryos, oocytes, and semen from animals that are potentially contaminated and where testing is not foolproof with these emerging diseases. More studies involving antivirals such as DB772, DAAs, and interferons are necessary to determine how efficacious these antivirals are at preventing detrimental effects of the virus on the embryo and prevention of disease transmission.

Summary

With the advent of synthetic media and antivirals, the embryo industry has made important strides in the prevention of transmission of diseases through ET. Continued evaluation of synthetic media and antivirals in culture systems for prevention of embryonic pathogens is imperative. These modalities might provide a reliable methodology to curtail infectious virus associated with embryos, oocytes, and semen in the future. However, proper biosecurity and quality control are always at the forefront of prevention of embryonic pathogens and should be the focus of standard operating procedures when utilizing any of the generations of embryo technologies.

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Cryopreservation of Bovine Embryos

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Introduction

Routine direct transfer (DT) of cryopreserved *in vivo* and *in vitro* derived cow embryos is not given a second thought today. It has become a common, integral part of genetic advancement and reproductive management of progressive beef and dairy herds. Data from the 2019 American Embryo Transfer Association (AETA) Statistics Committee reported that in 2018 of the 418 349 *in vivo* and *in vitro* derived embryos transferred in the USA (206 practitioners reporting), 179 810 (43%) were frozen [1]. Worldwide the percentage is much higher. Data from the International Embryo Technology Society (IETS) Data Retrieval Committee [2] indicates that of the 516 585 total *in vivo* derived embryos transferred worldwide in 2016, 321 022 or 62.1% were frozen, and 121 490 (37.2%) of the 326 623 *in vitro* fertilization (IVF) derived embryos transferred were frozen.

The convenience and success of transferring cryopreserved bovine embryos has made this routine practice possible. However, this has not always been the case. Prior to the development of reliable embryo cryopreservation techniques in the 1970s, transfer of cattle embryos from donors into recipient cows was limited to fresh recovered (not frozen) embryos transferred to available recipients on the premises or relatively close by. If more embryos were recovered than recipients were available for transfer, the remaining viable embryos were discarded [3]. In other cases, embryos would be chilled (4 °C) and personally transported in coolers on overnight commercial flights to other facilities where recipients were available. A reliable method of preserving cattle embryos was needed.

Modern embryo freezing technologies have evolved since the first reported successful transfer of frozen–thawed bovine embryos in 1973 [4] but didn't advance as quickly as developments for other procedures like embryo recovery and handling, transfer, superstimulation, and recipient management. Until the early 1990s glycerol was the most popular embryo cryoprotectant agent (CPA). While an improvement over previous systems, the main drawback of using glycerol as a CPA is that embryos had to be removed from the straws and glycerol removed from the embryo then reloaded into straws prior to transfer to recipients. In 1992, the method [5] referred to as direct transfer (DT) was introduced and was quickly adopted. Today in the USA almost 100% of the cryopreserved *in vivo* and *in vitro* produced embryos are frozen, thawed, and transferred using this method.

Cryopreservation

Cryopreservation of gametes and embryos is an essential procedure in advanced reproductive techniques for many species. Cryopreservation is the preservation of cells and tissues at subzero temperatures which essentially stops all biologic activity ("suspended animation") [6], preserving them for future use. Consequently, the viability of the preserved material after thawing is essential to the success of the procedure. Cooling mammalian tissues to extremely low temperatures exposes the cells to multiple risks: deleterious effects of cooling on cell functions and structures (chilling effects) [7], physical damage of cell membranes caused by transition of water to ice [6] (phase change transition), osmotic changes of cell dehydration, and exposure to CPAs [6, 7].

Most mammalian embryos are frozen in one of two methods: "slow rate freezing," equilibrium freezing prior to vitrification; or direct, ultra-rapid vitrification. The slow rate method allows for the embryo to equilibrate and

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

dehydrate prior to vitrification versus the direct vitrification process which does not include CPA equilibrium and cell dehydration. The equilibrium method of cooling includes slow, controlled reduction in temperature to allow extracellular ice crystals to grow and draw water from the cells without ice crystals forming within the cells. The slow rate of cooling is followed by vitrification (also referred to as plunging into liquid nitrogen [LN]) of the remaining intracellular water. The rate of cooling must be carefully controlled between 0.3 and 0.6 °C/minute [6, 7] to −30 to −32 °C. The remaining solute becomes more concentrated as more water molecules combine with the growing extracellular ice crystal. The hyperosmotic fluid outside the cells draws water from the cells, increasing the concentration of solutes and CPA within the cells. This also reduces the freeze point of the intracellular fluids which helps protect the cell from intracellular ice formation.

Vitrification is the rapid cooling of liquid into a "glass-like" state or the absence of crystal formation. During slow cooling, water molecules form a crystal structure as they rotate and form hydrogen bounds with four other surrounding molecules during the phase transition from water to ice. During vitrification, however, the freeze process is so rapid the molecules do not have the opportunity to form crystals. Vitrification is discussed in more detail in the section "Rapid Cooling."

Cryoprotectants

CPAs protect tissues and cells from injury caused by cooling to subzero temperatures and subsequent thawing. Embryos are placed into a hypertonic CPA solution (high concentration of CPA) to force water out of the cells, effectively dehydrating the embryonic cells prior to cooling. CPA compounds are either penetrating (act intracellularly) or non-penetrating (work extracellularly). Penetrating CPAs have small molecular weight and polar molecular structure similar to that of water so they readily cross embryonic cell membranes. For slow rate or conventional cryopreservation of bovine embryos, one of two penetrating CPAs are commonly used – glycerol or ethylene glycol (EG). Embryos are left in the CPA solution until they reach equilibrium [8]. As water leaves the cells, penetrating CPAs such as EG or glycerol enter the cell. EG reaches equilibrium in bovine embryos (5 minutes) more rapidly than glycerol (10 minutes) [8]. The high penetration and relatively low toxicity of EG make it especially favorable for DT applications [9].

Non-penetrating CPA compounds do not cross the cell membrane and work extracellularly. These compounds affect the osmolarity of a freezing solution and provide membrane-stabilizing properties. These compounds are generally sugars and include sucrose, galactose, and trehalose. These CPA compounds are not necessarily included in the freezing solutions utilized in slow-rate freezing but are frequently used to control the movement of water across the cell membrane after thawing (discussed in the section "Embryo Thawing"). Non-penetrating CPA compounds are commonly included in vitrification solutions. Freezing solutions used in slow-rate freezing commonly include only one CPA compound, though some practitioners do include sucrose with EG.

Modern freezing solutions for slow-rate freezing are typically commercially provided solutions. Penetrating CPA concentrations are normally 1.4 mol/l for glycerol or 1.5 mol/l for EG. The freezing solutions are hypertonic, causing cells to shrink on initial exposure but then slowly expand to their original size as the CPA enters the cells. Initially it was believed that this type of osmotic stress would reduce embryo viability and embryos were exposed to the CPA solutions in a stepwise manner. Empirical studies demonstrated that stepwise addition of CPA was not necessary. This is particularly true for EG. Embryos are placed in the freezing solution and allowed to equilibrate. Since 1.5 mol/l EG is not very toxic, equilibration times (5 minutes for 1.5 mol/l EG and 10 minutes for 1.4 mol/l glycerol [8]) are not critical and equilibration will continue while the embryos are loaded into 0.25-cc straws and during the first part of the cooling process. Embryos placed in a CPA solution will float because of the hypertonic nature of the solution and slowly sink as CPAs enter the cells. Normal practice is to start loading embryos as soon as they have settled to the bottom of the dish, with a minimum of 10 minutes of equilibration, before being placed in the embryo cooling chamber where they are held at −5 to −7.5 °C for 2 minutes before seeding (explained in the section "Ice Crystal Seeding").

Vitrification Solutions

In order for vitrification to occur at warm temperatures (20–25 °C), the CPA concentration must be higher than that described for slow-rate freezing. The majority of vitrification solutions used for bovine embryos consist of a combination of two or more penetrating CPAs and a non-penetrating CPA such as sucrose or trehalose. A typical vitrification solution for bovine embryos would consist of 15–20% EG, 15–20% dimethyl sulfoxide (DMSO), and 0.3–0.5 mol/l sucrose or trehalose. The combined concentration of EG and DMSO is at least three times the concentration of EG or glycerol used for slow-rate freezing. Extended exposure to these concentrations of CPAs can be toxic, so embryos are exposed to stepwise higher concentrations

and the exposure time to the final vitrification solution is limited to 40–45 seconds.

Rapid Cooling

In order for vitrification to occur at high temperatures, the cooling rate must be very rapid, in the order of 1000 °C/minute or greater. To achieve these rates of cooling, even by transfer directly into LN, the surface area to volume ratio must be greater than that of the 0.25-ml semen straw. One method of increasing surface area to volume ratio is the "open pulled straw" [10], which has been successfully used for the vitrification of bovine embryos. This is accomplished by gently heating the plastic semen straw and pulling it to yield a much smaller diameter and thus increasing the surface area to volume ratio. Additional methods of producing ultra-rapid cooling rates include the Cryoloop™, Cryotop™, and Cryoleaf™ [11]. These examples consist of various platforms or carriers that the embryo can be placed on in a minimal amount of vitrification solution. They create a high surface area to volume ratio and when placed directly in LN yield ultra-rapid cooling. All these carriers result in exposure of the embryo to LN, so once cooling is complete the embryo and carrier are placed in some sort of outer container that can be sealed. The same principle of high surface area to volume ratio that results in ultra-rapid cooling will result in ultra-rapid warming when placed in warm thawing solution. Thawing solutions will typically consist of a sucrose solution to ensure rapid heat transfer and control cellular swelling during removal of the CPAs.

Applications for Vitrification

Vitrification has been applied to the cryopreservation of human embryos and oocytes extensively, but far less for bovine embryos. At present there is no commercial application for cryopreservation of bovine oocytes and the value of vitrification for day 7 late morula or blastocyst stage bovine embryos has not been established. Vitrification has the advantage of not requiring a controlled-rate freezer and the freezing time for a single embryo is much less. However, the precise nature of the timing needed for vitrification requires that this procedure be conducted one embryo at a time. Because of this, when even a moderate number of embryos need to be processed, as much or more time will be required for vitrification compared with slow-rate freezing in which groups of embryos are frozen in a single cycle of a controlled-rate freezer. The precise nature of the timing and the handling procedures required during ultra-rapid cooling and warming during vitrification can result in variable success rates depending on the skill level of the person performing the procedure. There are very few data from controlled studies under practical conditions comparing success rates for vitrification compared with slow-rate freezing for late morula or blastocyst stage bovine embryos. Additionally, like embryos frozen in glycerol, thawing vitrified embryos requires removal of CPA and reloading into straws for transfer, making it less convenient than DT for bovine practice [12].

Embryo Selection, Handling, and Preparation

Once ova and embryos are removed from the reproductive tract of the donor cow they are placed in a commercially available isotonic holding media suitable to support embryo viability. If embryos are recovered from more than one donor laboratory, procedures should be in place to keep each sample separate from one another so that no embryos could be switched between petri dishes. This may include labeling all dishes with corresponding donor identification (both the dish and its cover should be identified) and embryo handling pipette tips should be changed between dishes. Ova and embryos are evaluated morphologically using a stereomicroscope with a minimum of at least 50× magnification and embryos and ova are segregated into unfertilized oocytes, degenerate embryos, and viable embryos. Viable embryos are further classified into grades one, two or three and degenerate embryos are classified grade four. The criteria for embryo grading, as well as the criteria for classification of embryonic stage, have been described in Chapter 9 of the *IETS Manual* [13].

Embryos are washed to remove any debris [13]. If embryos are intended for export the washing procedure should include two washes including trypsin. Chapter 6 of the *IETS Manual* covers sanitary handling of *in vivo* embryos in more detail. After sufficient washing, embryos are transferred into a CPA solution such as 1.5 M EG or 10% glycerol and individually loaded into 0.25-cc straws. The cotton/powder plug end of the straw is sealed when wetted by the fluid when the straw is filled during loading. The open end of the straw can be heat sealed or plugged and a label containing the registration numbers of the donor and sire, date of collection, breed, freeze code, and unique straw number is attached. Since embryo quality is a factor that varies considerably and has a major influence on the efficiency of cryopreservation, only grade 1 or 2 quality embryos are frozen. It is important to realize that many of the differences in morphological characteristics that constitute the embryo grading criteria are amplified by the freeze–thaw process. The freeze–thaw cycle can only be expected to reduce the number of viable cells, so these

characteristics that result in embryos being assigned lower grades are amplified by the freeze–thaw cycle. The pregnancy rate of grade 3 frozen/thawed embryos is typically unacceptably low. Embryos that have a damaged or missing zona pellucida also have reduced pregnancy rate following thawing and transfer.

Some practitioners fill the entire straw with 1.5 mol/l EG, while some load most of the straw with holding media and only the chamber containing the embryo with EG. There are numerous personal preferences to loading straws [14]. 0.25-cc straws can be inserted into the tip of a 1-cc tuberculin (TB) syringe for loading. The straw is rinsed to remove any fibers or plastic slivers before loading. A column of holding media or media containing 1.5 mol/l EG is aspirated followed by a short air chamber. The air chamber serves as a plunger and barrier to the cotton plug (if embryos contact the cotton plug they will not be expelled during transfer). Another short chamber of holding media is aspirated, followed by another air bubble; the embryo in EG is loaded into the straw, followed by another air bubble, and the rest of the straw is loaded with media. It is important that the air chambers be large enough to keep the chambers from mixing during freezing and thawing. The air chambers shrink during freezing and liquid expands so it is not uncommon for the chambers to move after the freeze–thaw process.

While stage of development is a factor in embryo cryopreservation for some species (human, pig, and horse, for example), embryonic stage is not a major factor for cryopreservation of bovine embryos within the context of normal embryo transfer. Differences in the ability to survive cryopreservation based on embryonic stage have been observed for bovine embryos [15], although all stages normally recovered from a day 7 (day 0 being observed or expected estrus) non-surgical embryo collection survive cryopreservation at an equal rate. A possible exception to this exists for hatched blastocysts, which have been observed to not survive cryopreservation well. Hatched blastocysts are not normally recovered at day 7 from cattle but are sometimes encountered when collection is delayed until day 8.

Embryo Freezing

The straws are placed upright (label end up and embryo in straw down) into a freezer chamber at −6 °C (range −5 to −7 °C) and held for two minutes prior to seeding (ice crystal formation; the instrument used to induce crystal formation should not be held near the embryo). The freeze program usually holds embryos at −6 °C for 6–10 minutes then cools at 0.5 °C per minute to −30 to −32 °C. Embryos are plunged into LN, packed into goblets labeled with the donor, sire, and date of processing information, and stored in an LN tank.

Currently, most practitioners freeze embryos in 1.5 M EG with or without sucrose which is the CPA used in the DT method of preserving embryos. No differences in pregnancy rates were observed between embryos frozen with or without supplemental sucrose in the CPA [14]. DT allows the embryo to be thawed, loaded directly into a transfer instrument, and transferred without rinsing the CPA or microscopic evaluation prior to transfer. Prior to the mid-1990s embryos were frozen in 10% glycerol. This method required embryos to be carefully deposited into a dish and moved through a series of dish wells containing decreasing concentrations of glycerol to remove the glycerol from the embryo, then microscopically evaluated for freeze/thaw damage prior to transfer. This procedure added time and required a clean "mini-lab" area on the farm to process the embryos prior to transfer. DT embryos are frozen and packed in yellow straws, goblets, and cane labels. Embryos frozen in glycerol are packed in white or clear goblets and white cane labels and should not be transferred in the direct method (personal experience).

It is critically important that embryos be handled with the utmost care. Embryos can be damaged or killed by mishandling, resulting in a reduced pregnancy rate. Embryos are susceptible to UV damage, chemicals (including osmotic changes), bacterial contamination, drastic temperature changes, and foreign materials (foreign bodies loaded and transferred with the embryo may result in a failed transfer). Implementing a total quality management (TQM) approach to embryo and laboratory hygiene to provide general cleanliness, aseptic techniques, and procedural consistency is critical to the success of an embryo production laboratory [13].

Ice Crystal Seeding

Aqueous solutions have a tendency to cool below their freezing point (undercool) before ice nucleation (freezing) occurs [16], which may result in spontaneous, uncontrolled ice formation including intracellular ice formation resulting in cell death. Control of when and where ice crystallization occurs is a necessary step in cryopreservation of bovine embryos. Ice crystal nucleation or "seeding" is intentionally inducing ice crystal formation in the extracellular media away from the embryo [6, 16, 17]. The straws are placed in the cooling chamber of the embryo freezer. After an equilibration period of two minutes in the chamber, ice crystal seeding is performed by touching part of the straw away from the embryo with metal forceps or a swab saturated with LN. Seeding induces ice crystal formation in the liquid in the straw and the ice formation spreads the length of the straw. As ice forms, water molecules are

removed from the liquid surrounding the embryos, increasing the solute and CPA concentration in the liquid around the embryo and drawing additional water molecules from the cells, which further dehydrates the embryo. The increasing concentration of CPA within the embryo reduces the freeze point of intracellular fluid [7], protecting the embryo from intracellular ice formation.

Embryo Packaging

The preferred package used for transferring and freezing embryos today is a 0.25-ml plastic straw [18]. The straws have an open end for loading and cloth wicks with a powder that seals the plug end when wetted properly. The ideal package containing the embryo should transfer heat and withstand changes in temperature from ambient room temperature to extreme cold storage (−196 °C) without damaging the container material and withstand rapid thawing from −196 to 30 °C. The package must remain intact and not allow any leakage of LN into the container. LN leakage may contaminate the contents of the straw and LN may vaporize quickly during thawing, potentially damaging the package and causing embryo loss. Semen and embryos are packaged in straws for convenience and efficiency of storage as well as ease of use in breeding and transfer pipettes.

Embryo Labeling and Record Forms

Confidence that given offspring resulted from the transfer of a specifically identified embryo is critical to the integrity of embryo transfer technology. Therefore accurate embryo recovery and labeling records are essential. Record forms have evolved to accommodate *in vivo* and *in vitro* embryo production as well as to transfer the information with embryos for international export. Chapter 9 of the *IETS Manual* [13] contains detailed information and instructions for proper labeling and record keeping as well as the proper forms (*ABC Embryo Certificates*) to provide a permanent record of the production, collection, and disposition of embryos.

The amount of information included on labels of cryopreserved embryos may vary. Minimal information should include the sire and dam of the embryo and the date the embryo was frozen. Additional information includes the practitioner or company conducting the freezing, breed of sire and dam, registration numbers, and the developmental stage and quality grade of the embryo at the time of freezing. The IETS provides "freezing codes" to its members that can be used to identify the practitioner or company conducting the freezing. This information should be printed directly on the label attached to the straw. Straw printing machines are available but not frequently used for embryos compared to semen straws because the information could be unique for each embryo. Information can be printed by hand but this can lead to legibility problems. One approach to labeling is to heat-seal a 0.5-ml straw to the 0.25-ml straw and apply labeling information on or inside the attached 0.5-ml straw. Another approach is the use of plastic identification and sealing rods. These rods attach to the end of a semen straw and can be used for labeling. In both of these approaches information can be printed on adhesive labels that can be wrapped around the labeling straw or plug. It is not recommended that labels be placed on the straw containing the embryo because this could interfere with the efficient transfer of heat during freezing and thawing. Another advantage of the approaches that place labeling information separate from the straw containing the embryo is that they provide a handle such that the information can be read without removing the straw containing the embryo from LN. Complete information about proper labeling of straws, goblets, and canes is provided by the AETA and can be found at https://www.aeta.org/docs/aetastrawlabel.pdf. Embryos frozen in EG are packaged in yellow straws. Non-DT embryos are in white. Embryos frozen by vitrification have blue labels or straws.

Embryo Thawing

Even though a slow rate of cooling is used in the early phase, a rapid rate of cooling is used in the final phase that results in vitrification of the remaining water in the cells. This results in a requirement for rapid thawing. If thawing is allowed to occur slowly, ice crystals will form during thaw and cellular damage will occur. Rapid warming is accomplished by transfer of the containers into warm (25–35 °C) water until all ice has thawed. It has been observed that if this rapid warming starts immediately from the liquid phase of LN there can be a high incidence of damage to the zona pellucida. Empirical studies have shown that if warming occurs in air for about 30 seconds before transfer to warm water, the incidence of zona pellucida damage is reduced. With this approach, warming through the critical temperature phase of above approximately −60 °C is still rapid and cellular damage due to ice crystal formation during thaw is avoided.

Often the thawing instructions are included at the bottom of the embryo processing certificate (*AC – Certificate of Embryo Recovery and Freezing* form). However, if the paperwork or other information is not available, the canes should be examined to make sure the embryos were frozen in 1.5 M EG designed for DT. If the embryos are not packaged in yellow goblets and straw labels or are not clearly labeled as DT they should not be thawed according to DT instructions. A commonly used procedure for thawing DT embryos is to air thaw for 5–10 seconds then submerse in 80–95 °F water for 15–20 seconds, wipe the straw dry, cut

the sealed end (the end opposite the cotton plug), load into a transfer pipette, and transfer into the recipient. A survey of embryo transfer practitioners in 1998 reported some variation of thawing temperatures with no differences in resulting pregnancy rates [14].

If embryos are frozen in glycerol the glycerol will need to be removed from the embryo prior to transfer. After thawing, the contents of the straw are carefully deposited into a petri dish and embryo(s) located and evaluated. During removal of glycerol, water enters the cells faster than glycerol exits and results in swelling of the cells. Cells are far less resistant to swelling than shrinkage and the degree of swelling that occurs with normal concentrations of glycerol can be very detrimental. Initially, this problem was circumvented by removal of glycerol by equilibration with stepwise lower glycerol concentrations. A number of approaches employing sucrose have been utilized, including a "one step" method using a 1-mol/l sucrose solution with an osmotic pressure equal to that of the freezing solution [19]. With this approach glycerol can leave the cells without water entering and no swelling occurs. After glycerol has been removed embryos can be placed in a physiological solution and water reenters the cells. Other approaches have utilized lower concentrations of sucrose [20], which can be removed in two steps. This approach prevents cellular swelling and also limits the degree of cellular shrinkage and dehydration. All these approaches have been successful in the preservation of viability of cryopreserved embryos but involve the movement of embryos between different solutions, requiring a certain degree of embryo manipulation skills and equipment between thaw and transfer. Sometimes the embryo has been severely damaged by the freeze/thaw process and judged unfit for transfer. It is not uncommon for the zona pellucida (clear envelope surrounding the embryo) to be fractured after thawing. As long as the cell mass is intact and resembles the description of the embryo prior to freezing the embryo should still be viable. The embryos are reloaded into straws with holding media, loaded into a transfer gun, and transferred into a recipient.

Transfer of Cryopreserved Embryos

The parameters for transfer of cryopreserved embryos to recipients are no different from those for transfer of non-cryopreserved or "fresh" embryos. Synchrony requirements between the donor and recipient estrous cycles are the same as for fresh embryos. This emphasizes the importance of including information concerning the stage of development on the labeling of cryopreserved embryos. If the embryonic stage is between a late compacted morula and a blastocyst, the assumption of a day 7 embryo is appropriate. Pregnancy rates for frozen/thawed embryos transferred to recipients 6.5–8.5 days after estrus are acceptable [21]. It is difficult to discuss expected pregnancy rates with any embryo transfer because there are so many variables. The same is true for the transfer of cryopreserved embryos. If high-quality day 7 bovine embryos are cryopreserved by the methods described here for slow-rate cooling, there should be an expectation of pregnancy rates very comparable to those expected for fresh embryos under the same conditions. The qualification of "high-quality embryos" is very important for cryopreserved embryos because the difference in pregnancy rates becomes significant for lower-quality embryos [22].

Embryo Freezers

There is a variety of embryo freezing equipment available, from inexpensive, manual machines that require constant manual adjustment and monitoring to more expensive, complex, computer programmed machines that require relatively minimal supervision. Freezing machines need to cool at the desired rate and the temperature in the freezing chamber should be monitored accurately. Some are designed to be used in LN (direct vapor or with the chamber suspended in nitrogen liquid) or an alcohol bath. Considerations for selecting equipment include reliability, ease of use and/or programming, number of straws or other types of containers, and whether equipment is portable or will remain in a laboratory. Regardless of expense and complexity, all equipment requires regular maintenance and supervision to ensure proper function. Be cautious of purchasing used equipment. Before purchasing, have the used equipment examined to make sure it is functioning properly and serviceable. A backup power source is essential. While some equipment has built-in battery backup, others may require some type of uninterruptible power backup source.

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Managing the "Problem Donor"

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Introduction

Genetic enhancement is a core motive for cattle producers, specifically seedstock producers. The bull can easily produce thousands of offspring through artificial insemination, and it has been, historically, more difficult to multiply the genetics of the female. These genetically superior females, or donor cows, are females whose oocytes or embryos are collected for transfer into a recipient cow in order to increase the number of offspring produced per donor. Genetics from females are mainly collected through one of two ways: ovum pick up (OPU) followed by *in vitro* production (IVP) of embryos or conventional embryo flush for embryo transfer (ET) [1]. In a conventional embryo flush, cows are inseminated following hormonal treatments to increase the number of ovulations per ovary in order to collect multiple embryos [2]. On the other hand, OPU can be utilized to collect oocytes from either exogenous hormone stimulated or non-stimulated donors [3]. Both techniques require investments of skill, time, and money; therefore selecting the ideal method that will provide the desired genetic enhancement can be a challenge. Criteria to select the correct protocol and approach for the donor cow includes factors such as age [4], subspecies (*Bos taurus* or *Bos indicus*) [5], body condition score (BCS) [6], and nutrition [7]. Donors' most common problem is being anestrous due to the presence of abnormal ovarian structures or the inhibition of hormones, which then would require individualized solutions [8]. Moreover, repeated exogenous ovarian stimulation by follicle stimulating hormone (FSH) can cause decrease in superovulatory response [9–14]. Variability in embryo quality and quantity is also one of the main problems found between donors and within the same donor. This chapter will discuss the factors that cause problems mentioned and management strategies to mitigate these challenges.

The Donor

Ovarian Dynamics of *Bos indicus* vs *Bos taurus*

B. taurus and *B. indicus* are subspecies of domestic cattle and have distinguishable physical characteristics with many physiological differences. *B. indicus* (zebu) cattle are known for their adaptation to tropical and subtropical climates [15], while *B. taurus* are more physically adapted to temperate climates [5]. In regard to reproduction, these subspecies have major differences in ovarian dynamics including follicular development and sensitivity to exogenous hormones that will affect ET and OPU outcomes. Understanding the physiology of each subspecies is essential to improve donor cow management.

Regardless of subspecies, cattle have a predominance of two and three follicular waves during each estrous cycle [16, 17]. Cows with two follicular waves will start the second follicular wave emergence later (day 9 or 10) than with three follicular waves (day 8 or 9) [18]. Considering that follicular wave pattern is repeatable within individuals [19] and that variability in treatments with exogenous hormones is dependent on the follicular stage [20], there is a need to synchronize emergence of a new follicular wave. The most common method to enhance superovulatory results is to synchronize the cow in order to coincide the initiation of exogenous superovulation treatment and wave emergence [18, 21], which will be addressed in the section "Variability in Response to Stimulation with FSH."

B. indicus cattle have greater small (<5 mm) antral follicle count [22] and greater percentage of oocyte recovery using OPU [23], even in the absence of superovulatory treatment [24]. When superovulatory protocols are used, *B. indicus* cows require lower quantities of gonadotropins to obtain the same number of oocytes recovered as *B.*

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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taurus [25]. *B. indicus* cows on average require 133–250 mg of FSH [26–31] while *B. taurus* require an average of 360–400 mg [27, 32]. Such response is due to the greater sensitivity to exogeneous FSH [15] suggested to be related to higher bioavailability of insulin-like growth factor 1 (IGF-1) in *B. indicus* follicles [33]; however, low quality oocytes remain problematic for donors of both subspecies. A better understating of oocyte quality will be addressed in the section "Variability in Embryo Quantity and Quality." Larger follicles have greater number of IGF-1 receptors regardless of breed [34–36] that aid in follicular growth [37] and oocyte maturation [38, 39]. *B. indicus* animals have smaller dominant follicles (10–14 mm) [16, 40–42] when compared to *B. taurus* (14–20 mm) [43, 44] which have higher concentrations of IGF-1. Nonetheless, *B. indicus* cows have a greater bioavailability of IGF-1 due to the elevated presence of the protein that degrades IGF-1 binding protein [33], indicating a potential mechanism for higher oocyte quality obtained from *B. indicus* cows compared to *B. taurus* [45], especially in tropical environments [46, 47]. Understanding ovarian dynamics such as follicular waves and most importantly characteristics inherent to subspecies like follicular size, oocyte quality, and sensitivity to exogenous hormones is key to utilizing and maximizing results from a hormonal or non-hormonal treatment involved in OPU and ET techniques.

Age

Cows are generally identified as potential donors when they have already produced a reliable reproductive and production record that demonstrates genetic superiority; however, there has been an increase in OPU and ET techniques performed in heifers selected for higher quality genetics. Nonetheless, in many cases, this decision comes later in life when the animal is older, leading to an interest in the association between age and donor quality which is often a negative relationship [48]. When making the decision to collect or flush a donor, the main objective is obtaining the greatest number of progeny in the shortest window of time to maximize lifetime productivity or to obtain superior genetics. In *B. taurus* beef cows 10 years of age and older, there is approximately a 20% decrease in the number of transferable embryos produced compared to younger cows [49]. Similar results were observed in Holstein and Charolais donors where total number of embryos and transferable embryos decreased as age increased [50, 51]. In contrast, others have reported no significant correlation between age and superovulatory response in Holstein, Simmental, and Angus breeds [51, 52].

Potential mechanisms of reduced embryo quality as age increases are yet to be established. Morphological abnormalities and differences in metabolic factors of the oocyte as age increases may contribute to future poor *in vitro* embryo development [53]. Another reason is attributed to the hormone concentrations before and during OPU in which younger animals had higher estradiol and lower progesterone levels [54]. High progesterone levels negatively affect follicle development in sheep [55], mice [56], and cattle [57] probably by the inhibitory effect on aromatase [58, 59]. On the contrary, older cows with lower progesterone levels before fertilization occurs have smaller ovulatory follicles and, thus, lower progesterone levels after ovulation and a decrease in embryo quality [4]. Moreover, oocyte abnormalities are higher in older cows due in part to a high incidence of failure to extrude the second polar body in the zygote (i.e. one-cell embryo) caused by impaired nuclear maturation [60, 61]. Lower oocyte quality due to an increase in age is also associated with a decrease in gene expression of the protein quality control system and genes related to mitochondrial activity [62]. Overall, in most cases, donor cows are older animals which can still produce good quality embryos and oocytes, indicating that age is not a decisive factor in the use of ET or OPU techniques; however, age can negatively impact oocyte and embryo yields.

Body Condition Score (BCS)

All living beings allocate available energy in a specific order, beginning with basal metabolism and growth, while estrous cyclicity and reproductive performance have the lowest priority [63]. BCS is a tool used to indicate the nutritional status of cows by visualizing the amount of fat and muscle in certain areas. Poor body condition will result in reproductive system quiescence to preserve proper function of other vital systems (the central nervous system, cardiovascular system, etc.). If donor cows are overfed and maintained at a high body condition this will normally negatively impact superovulatory response, decrease embryo and oocyte quality, and reduce pregnancy rates [64, 65]. High body condition decreases IGF-1 follicle concentrations [66], thus reducing the sensitivity of granulosa cells to exogenous FSH treatment and negatively affecting oocyte maturation [33, 67]. Moreover, cows with high body condition are predisposed to hyperinsulinemia and create an insulin resistance state, which impairs fertilization and embryo development [48, 68].

Produced by adipose tissue and increased by food intake, the hormone leptin is believed to be a key regulator of reproductive function. Increased circulating leptin has been positively correlated with luteinizing hormone (LH) pulses [69, 70] and leptin concentrations in follicular fluid [71]. Additionally, supplementing leptin to *in vitro*

maturation media increased subsequent blastocyst development and reduced cell apoptosis [72]. In spite of its advantages, higher body condition related to obesity and elevated concentrations of leptin can create a resistance to leptin in mice and women [73, 74]. Murine and bovine oocyte development and, thus, embryo yields *in vitro* are impaired by high levels of leptin [75, 76]. On the contrary, animals with low BCS have reduced plasma leptin levels [77]. Moreover, a decreased BCS and high energy requirements lead to a negative energy balance which increases non-esterified fatty acids (FAs) in the system [78], including in follicular fluid. Such levels may cause negative effects on oocyte maturation and thus on subsequent fertilization and embryo development *in vitro* [79]. In summary, ensuring good body condition in the donor cow will improve oocyte and embryo quality results; however, in cases where the animal is underweight, an OPU can be implemented and have better results than an ET.

Nutrition

Nutritional status will either positively or negatively affect reproductive performance, especially regarding the development and recruitment of follicles, and subsequent oocyte and embryo development. Thus, an animal's plane of nutrition must be intensely, yet carefully managed. In postpartum, lactating donor dairy cows, changes in metabolism and nutrient demands result in negative energy balance [80]. Energy deprivation reduces the concentration of IGF-1 and insulin by uncoupling of the growth hormone–IGF system, and reduces LH pulses, impairing follicle maturation and ovulation [81–83]. Moreover, excessive body fat mobilization is correlated with increase non-esterified FA and beta-hydroxybutyrate in follicular fluid [84, 85], which increases oocyte cell apoptosis and impairs early embryonic development [86–90]. Providing a diet with adequate energy concentration can overcome these endocrine changes and re-establish follicular fluid estradiol, improving oocyte quality during the postpartum period [91, 92].

Short- and long-term changes in energy intake affect superovulatory response in lactating and non-lactating donor cows. In lactating donor cows with lower body condition, increases in energy intake to levels higher than maintenance requirement elevate circulating insulin and IGF-1 [48, 68, 93]. These growth factors improve the effect of gonadotropins on follicular granulosa and luteal cells, enhancing follicle recruitment and steroidogenesis [38, 94]. In non-lactating donor cows with adequate or high body condition, however, high energy intake can increase catabolism of steroid hormones and high circulating concentrations of insulin and free FA derived from adipose tissue [68, 87, 95, 96]. High insulin and free fatty acid concentrations will most likely create an insulin resistance state and impair follicle stimulation response [48, 97, 98]. Overfeeding non-lactating cows or heifers, therefore, may reduce superovulatory responses.

Supplementation with fat, protein, and minerals can enhance oocyte and embryo quality. Results of fat supplementation in the form of FA are dependent on the lipid source (saturated or unsaturated) [99, 100]. Fat supplementation with unsaturated FA (not degraded in the rumen) as a major component can improve quality of *in vitro* produced bovine oocytes [101] and embryos [102], and porcine embryo viability after cryopreservation [103]. Bovine embryo quality is enhanced possibly by an increase in trophectoderm cells in the blastocyst stage [102]. Likewise, protein supplementation can improve reproductive performance; however, providing protein above nutrient requirements can lead to increased levels of urea (metabolite of protein digestion in the rumen) in the system [104, 105]. High levels of urea impair oocyte and embryo development by disrupting meiosis, as this also lowers oviduct and uterine pH, affecting embryo development and fertility [106, 107]. Mineral supplementation is a crucial yet often overlooked management tool that can affect embryo production. Recovery of cumulus oocyte complexes from donors supplemented with organic trace minerals improved subsequent *in vitro* embryo production when compared to those donor's receiving inorganic mineral sources [108]. Donors in good nutritional status and body condition, however, may not see an added benefit from organic trace mineral supplementation [109].

Adequate nutritional status can enhance reproductive performance in donor cows by improving embryo and oocyte quality. Managing high-energy diets must take into consideration the animal's metabolic status and body condition, with overfeeding constantly having detrimental effects. Supplementing FA or mineral is dependent on its source, while the negative effects of high-protein supplementation are related to urea circulating concentrations. In summary, donor cow's diets must be formulated to ensure a level energy balance combined with good body condition; moreover, it is important to supply nutrients that will optimize and meet the animal's requirements.

Problems Related to Donors

Anestrous Donors

Anestrous is a period where normal ovarian cyclicity ceases, causing an absence in estrus behavior. Heifers have a natural anestrous period until the onset of puberty and

pregnancy, which is also considered an anestrous stage [110, 111]. Naturally, cows experience a 40- to 60-day period of anestrous after calving [112–114] and management decisions can increase or decrease the length of this period. Additionally, significant research has evaluated voluntary waiting period (time from parturition to first insemination) management strategies in dairy herds to maximize reproductive efficiency [115, 116]. Extended postpartum anestrous can be attributed to two main causes: (i) uterine disease and infection and (ii) insufficient nutritional status prior to and post parturition. Uterine infections will increase the time required for complete uterine involution after parturition, in which the uterus shrinks back to its normal size [117]. Meeting nutritional requirements of the cow to prevent negative energy balance postpartum and ensuring a good BCS will aid in normal uterine involution and return to cyclicity by allowing the hypothalamus to resume its secretion of gonadotropin releasing hormone (GnRH) [118]. Considering the risk of long anestrous intervals and cyclicity failure, it is important to ensure proper management of the donor cows by maintaining good body condition at least one month prior to any ET or OPU procedure. If requirements are not met, deficiencies in protein quality, feed intake, and mineral, vitamin, and energy requirements must be addressed.

Persistent ovarian structures, including follicular cysts and corpus lutea, are a common cause of anestrous periods associated with a negative energy balance after parturition in high-producing dairy cows [8]. During the first cycle following parturition, 6–30% of dairy cattle will develop an ovarian cyst [119, 120]. A persistent follicle is considered cystic when it has a diameter greater than 17 mm and it persists for over six days [121]. Advances in technology, such as ultrasonography, allow visualization of follicular and luteal ovarian dynamics contributing to easier management of cows with persistent structures [122]. Symptoms of follicular cysts include altered estrous cycles and shorter intervals between estrus manifestation that aid in identifying problematic cows [123, 124]. Literature commonly characterizes altered LH release from the pituitary by GnRH as the main cause of an ovarian follicular cyst mainly due to a distorted feedback on the hypothalamus [8, 125–127]. Although cysts can undergo regression without interference, physical manipulation such as rupturing the follicle while performing a transrectal palpation or exogenous hormones such as GnRH or progesterone will eliminate the cystic follicle [128]. Performing a manual rupture of the cystic follicle should be carefully regarded as hemorrhaging may result [129]. Administration of GnRH will induce a surge of LH to cause ovulation of the cystic follicle [130]. Progestin devices have also shown to effectively regress follicular cysts, prevent future development of others, and return cyclicity in beef cows [119, 131, 132]. Alternatively, other data indicated that progestin-based estrus synchronization when used over long intervals of time may increase the risk of follicular cyst development as a result of the presence for longer periods of the dominant follicle [133]. Another solution for cystic follicles is treatment with human chorionic gonadotropin (hCG) [134] or addition of hCG to the synchronization protocol [135]. Treatment with FSH will not cause a regression of the follicular cyst but increases LH receptors that will aid in responsiveness of granulosa and luteal cells to induce ovulation [136].

Cysts can also be characterized as luteal cyst, with the main differences between them being the deficient luteinization process in a follicular cyst and wall thickness, with luteal cyst having a wall larger than 3 mm and it can be visibly seen in an ultrasound as a white or gray color [8]. Luteal cysts secrete progesterone in higher concentrations and are bigger in size than a follicular cyst [120]. These structures are corpus luteum (CL)-like in terms of production of progesterone and luteinized cells, so prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration results in the regression of luteal tissue [137, 138].

An inability to have follicles differentiate into a dominant follicle or anovulation of the dominant follicle can lead to an extended anestrous period. Treatment with progestins has shown to be advantageous with anovulatory or unresponsive ovaries. A progesterone releasing intravaginal device (PRID) is an exogenous, non-invasive way to administer therapeutic progestins. Such protocols normally utilize $PGF_{2\alpha}$ coupled with estradiol or GnRH doses in countries where estradiol is banned from use following progestin removal [113]. The mechanism of combining these hormones to aid in dominant follicle development is by suppressing ovulation with progesterone or maintaining an existing CL, then removing the negative feedback of progesterone and regressing the CL by administering $PGF_{2\alpha}$. The subsequent administration of GnRH or estradiol will induce a new follicular wave formation and, thus, a new dominant follicle and estrous cycle. A more overt or more strongly expressed estrus has been shown to be associated with higher embryo viability [139]. In general, an extended anestrous stage in cows can be prevented by ensuring good nutrition and adequate body condition. Moreover, impaired estrous cyclicity in donor cows caused by abnormal ovarian structures can be detected based on clinical signs or with an ultrasound examination and treated with exogenous hormones.

Repeated Superovulatory Treatments

Use of exogenous hormones to stimulate follicular development and growth in cows has been well documented [18, 140]. Superovulatory protocols can be performed every

30–40 days for ET with no detrimental effects on oocyte yield or quality [140, 141], but a decrease in response is observed after three to five sessions [52, 142–144]. Following repeated superovulatory procedures, the effectiveness of FSH stimulation will decrease, which can be explained, in part, by an alteration of the FSH receptor [9]. Therefore donors should be rested for an extended period, allowing the opportunity to carry a natural calf or to establish pregnancy and induce abortion.

In rats, cows, and pigs, a continuous dose of FSH results in desensitization of FSH receptors in granulosa cells [10–13, 145]. Treatment with exogenous progesterone and $PGF_{2\alpha}$ between superovulatory protocols may reduce detrimental effects of stimulation by preventing ovulation and inducing luteolysis [2]. Additionally, cows with low stimulatory response at the beginning of the treatment may be prescribed higher FSH doses in subsequent protocols to increase stimulation [146, 147]. Summarizing, detrimental effects on follicle stimulation response by repeated superovulatory treatments can be nullified by providing a resting period after three to five ET sessions, establishing pregnancy and inducing abortion, or with exogenous hormone treatment between superovulatory protocols.

Variability in Response to Stimulation with FSH

As previously mentioned in this chapter, FSH is the hormone responsible for the stimulation of recruitment and development of ovarian follicles. Due to the short biological half-life of FSH in the bovine circulatory system (approximately 4–6 hours), a typical superovulation protocol involves a series of 7–10 separate FSH doses administered at 12-hour intervals over a 4-day period [148, 149]. Typically, 72 hours after initiation of the superovulation protocol, $PGF_{2\alpha}$ is administered to induce luteolysis (regression of the CL) and enable onset of estrus (36–48 hours) and ovulation (24–36 hours) in donors [150]. The dose of FSH will vary based on product, body weight, stage of production, and the breed of the donor. Heifers and *B. indicus* cattle have a greater sensitivity to FSH and, thus, typically receive a lower dose of FSH for superovulation [21, 151]. While the dose of FSH varies, it is important to note that too much exogenous FSH negatively impacts the superovulatory response by causing the donor to produce the ovarian hormone inhibin, which will inhibit FSH production by the pituitary gland [152, 153].

Individual variation among donors in superovulatory response and the reasons for variation are not well understood. Previously mentioned intrinsic (e.g. age, genetic differences, BCS) and extrinsic (e.g. nutrition, hormone preparation) factors do influence the superovulatory response. The ovarian status (i.e. phase of the estrous cycle) of the donor at initiation of the superovulation protocol has been suggested to be a factor determining the superovulatory response [154]. There have been inconsistent reports on the effect of the presence of a dominant follicle at the time of initiation of the superovulation protocol on superovulatory response; however, multiple studies agree that the presence of a functional dominant follicle (i.e. diameter of >9 mm and stable diameter for less than 4 days) may be a limiting factor for superovulatory response [155]. One method to ensure the absence of the dominant follicle is ablation of the largest/dominant follicle prior to the superovulatory protocol [156, 157]. The results are inconsistent and this technique requires equipment and trained personnel for ultrasound-guided follicle aspiration. It is a common practice to initiate the superovulation protocol 8–12 days (i.e. mid-cycle) after natural estrus expression [18]. This is an ideal time for initiation because it encompasses the time of the emergence of the second follicular wave in cattle exhibiting two- and three-wave cycles [158, 159]. Moreover, many investigators have found a positive correlation between the number of small (2–4 mm) and medium (5–8 mm) antral follicles present on the ovary prior to superovulatory treatment and a better superovulatory response including the number of corpus lutea after superovulation and the number of transferable embryos recovered [160–162]. In contrast, others have not identified any correlation between the number of follicles and superovulatory response [163, 164]. Taken together, initiation of the superovulatory protocol during mid-cycle can be beneficial, and the quantity and quality of follicles that can respond to FSH is the primary reason for the variability of superovulatory response between individuals.

Variability in Embryo Quantity and Viability

The number of embryos recovered from a donor is directly dependent on the number of follicles that ovulate. While superovulation produces more follicles and thus more oocytes, the superovulation procedure may induce abnormalities in oocyte maturation, which negatively impacts proper fertilization and/or embryo development. This can result in the subsequent recovery of unfertilized oocytes or poor-quality embryos.

To understand the potential negative impacts of superovulation on oocyte maturation, it is important to know the normal morphologic changes occurring during oocyte maturation *in vivo*. Oocyte maturation begins prenatally, and oocytes must undergo two meiotic divisions (meiosis I and II) [165]. The oocyte is arrested at prophase I and will resume meiosis once the follicle reaches dominance [166]. The LH surge controls the process of oocyte maturation (i.e. nuclear and cytoplasmic maturation) and resumption

of meiosis, and the oocyte will arrest at metaphase II until fertilization [167, 168]. It has been demonstrated that superovulated cattle display abnormal oocyte maturation based on the stage of nuclear and cytoplasmic maturation and time at which the LH surge occurred. Hyttel et al. [169] found that 37% of oocytes collected from superovulated cattle were classified as having undergone abnormal oocyte maturation. This would increase the likelihood of recovering unfertilized oocytes or poor-quality embryos. Typically, fertilization rates are 85–95% in cows with a single ovulation and it has been shown that fertilization rates in superovulated cows are reduced to 65–70%, most likely due to the altered oocyte maturation and suboptimal oocyte quality [155]. Blondin et al. [170] conducted a study to establish an ideal superovulation protocol that would maximize the number of competent oocytes (i.e. those that had undergone proper oocyte maturation and subsequent embryo development) collected from Holstein heifers. Treatments included four versus six FSH doses, collecting oocytes via OPU either 33 or 48 hours following the last dose of FSH, and administering LH 6 hours before OPU or not. Collected oocytes were subjected to *in vitro* fertilization to assess oocyte competence and embryo production rates. Results indicated that six FSH doses and performing OPU 48 hours following the last FSH dose greatly improved embryo production; however, administration of LH was more beneficial when OPU was performed 33 hours following the last FSH dose (Table 88.1). Together this suggests that inducing a preovulatory-type follicular environment (i.e. OPU 48 hours following final FSH dose plus LH administration) allows for proper nuclear and cytoplasmic maturation and oocytes are developmentally competent, thus improving subsequent embryo development.

While the number of embryos produced from a donor is important, perhaps the most critical aspect relative to successful ET pregnancy establishment is the quality of embryos transferred. Embryo quality is determined by visual assessment of the morphological characteristics of the embryo and a grade is assigned (grade 1 = excellent or good, grade 2 = fair, grade 3 = poor, grade 4 = dead or degenerating) following International Embryo Transfer Society guidelines. Unsurprisingly, pregnancy rates are greatest when grade 1 embryos were transferred compared to grade 3 embryos (50–60 vs 30–40%, respectively) [171]. While advancements have been made to optimize superovulation protocols, the number of transferable quality embryos (grades 1 and 2) recovered from donors has seen little improvement. A recent study evaluated the effect of circulating progesterone (high vs low) during a traditional superovulation protocol on embryo quality [172]. While the total number of embryos recovered was reduced for low progesterone donors, the percentage of grade 1 embryos recovered was significantly increased for low progesterone donors compared to high progesterone donors (58.2 vs 37.3%, respectively). Moreover, high progesterone donors had a greater percentage of grade 3 embryos and unfertilized oocytes recovered. A few studies that have evaluated the role of progesterone during follicular development have found that low progesterone results in increased LH pulse frequency and estradiol concentrations, but did not affect oocyte competence [173–175]. It appears that the combination of the endocrine profiles associated with low progesterone during follicular development and FSH doses during the superovulation protocol improves oocyte competence and subsequent embryo development. Therefore it may be advantageous to utilize a low or subluteal progesterone protocol (i.e. administration of $PGF_{2\alpha}$ plus exogenous progesterone at the start of the superovulation protocol) for donors that repeatedly produce poor quality embryos.

Table 88.1 Effect of collecting oocytes via OPU either 33 or 48 hours following the last dose of FSH, and LH administration (with or without LH), with four or six FSH doses on *in vitro* embryo production.

	Treatment		Blastocyst rate (%)[a]	
Number of FSH doses	**33 vs 48 hours**	**LH administration**	**Day 7**	**Day 8**
4	33 hours	No	20	40
4	33 hours	Yes	54	67
4	48 hours	No	43	53
4	48 hours	Yes	41	54
6	48 hours	No	52	63
6	48 hours	Yes	63	80

Source: Adapted from [170].

[a] Blastocyst rate is the percentage of embryos produced from an inseminated oocyte.

An Alternative for the Problem Donor: *in vitro* Production of Embryos

In the early 2000s, IVP of embryos emerged as an alternative to superovulation for embryo production from donor females [176, 177]. There are some advantages to IVP, including subfertile and pregnant donors can still be subjected to OPU; donors can be subjected to OPU at frequent intervals to increase the yield of embryos produced; efficiently using sexed semen; and rapid utilization of multiple sires [178–180]. One major advantage of IVP is the ability to collect a greater number of oocytes without the dependency on exogenous gonadotropin stimulation. This advantage overcame one of the major issues of conventional ET, which is an inconsistency in ovarian response to superovulation. In *B. indicus* breeds, this advantage is even more pronounced because a greater number of follicles are present on the ovary throughout the estrous cycle [24]. It has also been shown that the antral follicle number plays a role in oocyte recovery [181].

With these advantages of IVP, a greater number of transferable embryos per donor can be generated through IVP than through superovulation over time; however, the efficiency of IVP is low, with only 10–40% of oocytes developing into transferable embryos [182]. Moreover, pregnancy rates are approximately 10% lower for IVP embryos compared to superovulation derived embryos [183] and pregnancy losses are greater for IVP embryos [184, 185]. Additionally, pregnancy establishment of IVP embryos is more embryo stage and quality specific than *in vivo* derived embryos. Greater pregnancy rates were achieved following transfer of expanded blastocysts (stage 7) compared to morula (stage 5) and early blastocysts (stage 6) [186, 187]. Furthermore, while both grade 1 (excellent embryo quality) and 2 (good embryo quality) embryos produced *in vivo* can be transferred with no differences in pregnancy rates, transferring grade 2 *in vitro* produced embryos will result in a greater chance of early embryonic loss during the first 14 days after transfer [188].

IVP of bovine embryos has gained worldwide interest, with 58% of the ET performed in 2017 being IVP embryos [189]. Regardless of the setbacks and hurdles that IVP systems pose, the benefits of utilizing this tool cannot be ignored. Specifically, with this technology, donors can produce more offspring each year than by conventional ET and problematic donors can be used. However, from a management perspective it is important to note that some cows respond well to OPU/IVP but not ET, and vice versa. Therefore identifying the ideal scenario for each donor will help to increase genetic proliferation.

Summary

In managing problematic donor cows, the practitioner must consider the interaction between factors that may affect superovulatory response to exogenous hormone stimulated or non-stimulated techniques, thus influencing embryo and oocyte quality and viability. Intrinsic factors such as subspecies (*B. indicus* vs *B. taurus*), age, and body condition and extrinsic factors like nutrition contribute to this response. Older cows and body condition at either extremes (too fat or too thin) will negatively impact oocyte and embryo quality, while subspecies influences the sensitiveness of the exogenous hormone treatment response. Most common problems related to donor cows are being in anestrous, lower response to superovulatory treatment after repeated protocols, variability in response to FSH doses, and embryo and oocyte inconsistency in quality and quantity (Table 88.2). Solutions to such problems vary according to the animal and cause of the problem;

Table 88.2 Definition, causes, and suggested solutions of problems related to problematic donor cows.

Problem	Definition	Causes	Suggested solution(s)	References
Anestrous donors	↓ normal cyclicity and estrus behavior	Cystic follicle Luteal cyst	Ablation, exogenous GnRH or hCG, progestins, synchronization protocols, $PGF_{2\alpha}$	[121, 128–132, 134, 135, 137, 138]
Repeated superovulatory treatments	↓ superovulatory response	↓ FSH receptor	Resting periods, progesterone, and $PGF_{2\alpha}$	[2, 52, 142–144]
Responses to FSH	Variable response to FSH treatments	Intrinsic and extrinsic factors	Initiate superovulation mid-cycle, ablation of dominant follicle, cow selection with ↓ antral follicles, IVP	[18, 156–162]
Embryo quantity and quality	Variable production of good quality embryos	FSH doses and various endocrine profiles	$PGF_{2\alpha}$ + exogenous progesterone at initiation of superovulation	[170, 173–175]

however, for anestrous cows a variety of exogenous hormone treatments can return and prevent acyclicity. The negative effect of repeated superovulatory treatments can be nullified by allowing the animal a resting period to carry a normal pregnancy. Moreover, variability to FSH treatments can be decreased by initiation of superovulatory treatment in mid estrous cycle, ablation of dominant follicle prior to treatments, or performing OPU and *in vitro* fertilization techniques. Lastly, variability in embryo and oocyte quantity and quality can be altered by a dose of $PGF_{2\alpha}$ and exogenous progesterone at initiation of superovulation treatments.

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In Vitro Fertilization

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Introduction

In Latin, *in vitro* means "in glass," and *in vitro* fertilization (IVF) is frequently used as a general term for the process of generating embryos outside of the body, which also includes *in vitro* maturation (IVM) and *in vitro* culture (IVC). Typically, these procedures are conducted in the sequence IVM–IVF–IVC to produce embryos exclusively *in vitro* (IVP). Glass is rarely used for *in vitro* procedures today, having been replaced by disposable plastics, primarily made from polystyrene or polypropylene.

In 1959, the rabbit was the first mammalian species in which live offspring were produced by IVF [1], followed by laboratory mice in 1968 [2]. Because the mouse was not a good model for cattle, the first successful IVF with cattle was in 1977 when sperm were capacitated in the oviduct or uterus of a cow or rabbit [3] and the first live calf born in 1981 when a four-cell embryo was transferred into the oviduct of a recipient cow [4]. The first calves produced entirely from IVM, IVF, and IVC were born in 1987 [5]. Since that time, research on *in vitro* procedures has grown dramatically. In addition, *in vitro* procedures are now being used commercially in conjunction with embryo transfer (ET) in a number of countries. In this chapter we intend to describe the most current techniques for oocyte collection, IVP of embryos, and the utilization of IVP embryos in the cattle industry. Some of the applications of IVF technology in cattle are listed in Table 89.1.

Collection of Oocytes

Originally, *in vitro* procedures in cattle were conducted primarily for research purposes and utilized oocytes collected from superovulated females. When collected from preovulatory follicles or oviducts 20–24 hours after onset of estrus, oocytes have already undergone maturation and are ready immediately for IVF, followed by an additional interval for IVC. Oocytes collected from slaughterhouse-procured ovaries require IVM; thus research utilizing these oocytes lagged until IVM techniques were improved. Today, ovaries from cattle slaughterhouses are used extensively as a source of oocytes.

Oocyte Collection from Living Cattle

The primary method of collecting oocytes from live cattle is aspiration of ovaries manipulated per rectum and guided by a vaginally inserted ultrasound probe and needle. Prior to the development of this technique, oocytes were obtained surgically through a flank incision [6] or laparoscopic procedures via the paralumbar fossa [7], though these approaches were expensive, inefficient, and risked the formation of adhesions with subsequent loss of fertility. The history of laparoscopic oocyte recovery is reviewed by Tervit [8].

The introduction of real-time transrectal ultrasonic imaging [9] led to the development of techniques for the repeated collection of oocytes from bovine females. Ultrasound-guided collection of oocytes via the paralumbar fossa was described in 1987 [10], and the first repeatable, efficient technique involving transvaginal ultrasound-guided aspiration was developed in 1988 [11]. This technique has become widely known as ovum pick-up (OPU) or transvaginal aspiration (TVA). An ultrasound transducer and an attached needle guide of the type used in cattle are shown in Figure 89.1. A diagram of how the transducer is inserted into the vaginal fornix so that a needle can be guided through the vaginal wall and into the ovary is also shown. Today a large number of appropriate

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine

Table 89.1 Applications of IVF technology.

Commercial applications
Offspring from infertile cattle
Offspring from pregnant cattle
Offspring from young heifers prior to breeding age
Salvage of genetics from terminally ill/injured cattle
Efficient use of sexed semen
Use of resorted semen (frozen–thawed and sexed after thawing)
Use of multiple sires in a short period of time
Utilization of slaughterhouse-derived oocytes for production of research and/or inexpensive embryos
Research applications
Improvement of IVF technology
Improvement of IVC for cloning and transgenic procedures

ultrasound monitors and transducer/needle collection devices are available from different manufacturers for OPU.

The number of oocytes collected from a cow during a single session of OPU depends on a variety of factors. On the mechanical side, the vacuum pressure used to remove the oocyte from the follicle, the gauge of the needle [12], and the length of the bevel on the needle [13] all impact the number of intact cumulus-oocyte complexes collected. Frequency of collection is another factor. OPU can be performed once or twice a week on the same cow without the use of exogenous hormones. Studies have demonstrated no advantage of interval length for the number of oocytes retrieved per collection when three, four, or seven days were compared [14–16]. The ultimate goal is to generate embryos that can be transferred to recipients, and from that perspective there is evidence that a three- or four-day interval (8 oocytes per OPU) is better than a seven-day interval (5.5 oocytes per OPU) [17].

The number of oocytes collected per OPU can be increased by pretreatment of the donor with gonadotropins [18, 19]. In a very comprehensive comparison of different OPU intervals with and without follicle-stimulating hormone (FSH), Chaubal et al. [20] reported that the most productive protocol in terms of oocyte and embryo production involved dominant follicle removal, FSH treatment 36 hours later, and OPU 48 hours after FSH. This treatment was alternated weekly with simple once-weekly OPU. The OPU weekly session involving FSH averaged 10.6 oocytes and 2.4 blastocysts, while the alternating weekly OPU with no FSH averaged 4.6 oocytes and 0.9 blastocysts. The interaction of factors, including the frequency of OPU and the inclusion or absence of FSH stimulation, has been described in a number of studies, including some published in 2002 [21] and 2008 [22]. Since then, a number of publications have clearly demonstrated that the use of FSH prior to OPU increases the number of usable embryos at the end of IVP [22–28]. Also, a study in Japan [29] utilizing both Holstein cows and Japanese Black cows showed that a single injection of FSH or 4 injections at 12-hour intervals resulted in a higher percentage of both grade 1 oocytes than an injection of gonadotropin-releasing hormone (GnRH) or no pretreatment prior to OPU. Also, a single injection of FSH resulted in a higher proportion of blastocysts than the other treatments. Most surprising perhaps, the conception rate following ET was higher in the FSH

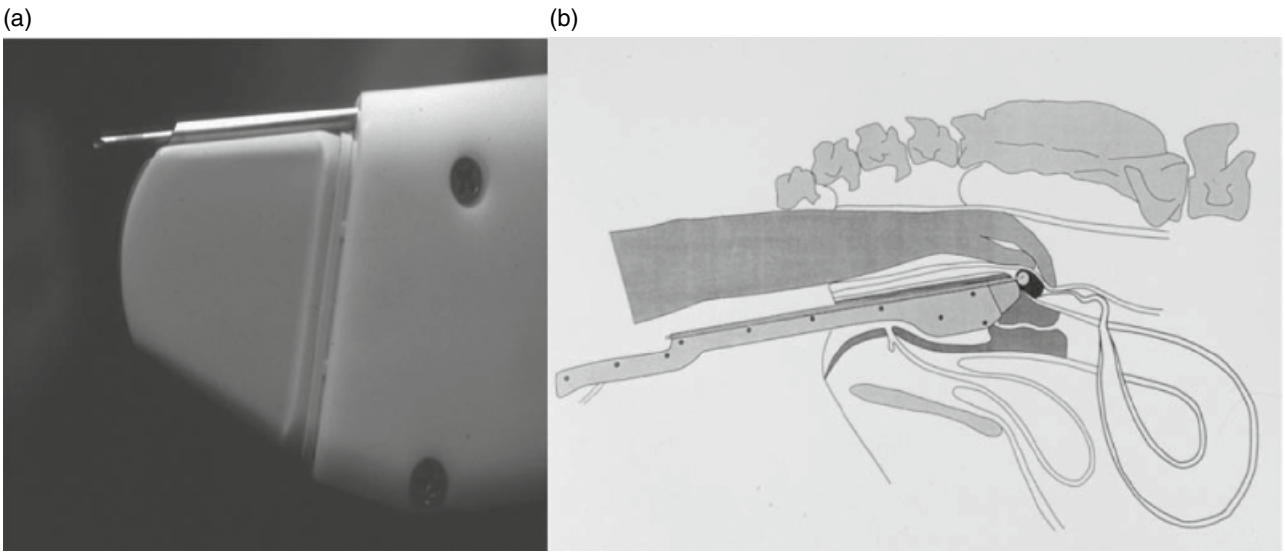


Figure 89.1 (a) Ultrasound transducer with oocyte aspiration needle protruding from needle guide. (b) Diagram showing position of ultrasound transducer pressed against vaginal fornix, with ovary manually manipulated per rectum and held up against vaginal wall.

pretreated donors. However, at least two studies failed to find an increase in the number of embryos produced as a result of FSH stimulation in non-lactating Holstein cows [29, 30]. Additionally, it has been shown that multiple injections of FSH were no more effective in increasing the number of embryos following OPU and IVP than when a single injection of FSH dissolved in 0.05% hyaluronan was used [31].

Breed of cattle also is an important factor. In South America (primarily Brazil) large numbers of oocytes are routinely collected with no FSH priming from Nelore cattle, an indigenous breed of *Bos indicus* origin known to develop higher numbers of follicles in each follicular wave than *B. indicus* females [32]. There are many unpublished reports of oocyte numbers following OPU ranging from several to more than 500 in these cattle. Furthermore, it was shown in Brazil that more embryos and pregnancies could be produced per unit time from *in-vitro* derived embryos compared to *in vivo* embryos derived from repeated superovulations [32]. In unstimulated *Bos taurus* cattle in the USA, low mean numbers of usable oocytes ranging from 4.1 to 5.3 were reported by Hasler et al. [33] and Looney et al. [34] from combined populations of dairy and beef breeds, including older females with a variety of fertility problems.

The concept of coasting can be defined as the time period between the cessation of FSH injection(s) providing stimulation of multiple follicles and the harvesting, usually via OPU of the oocytes. Much of the pioneering work involving coasting was conducted in Canada [21, 35]. In further work in Canada on this concept, "coasting" was also described as FSH "starvation" [36]. In this study the highest rates of blastocyst development *in vitro* were obtained when FSH support was discontinued for 44–68 hours and granulosa cell SMAD7 and mRNA were predictive of this period. The coasting procedure that is widely used today in the commercial IVP industry involves a range of approximately 30–44 hours between the last FSH injection and OPU. The use of FSH with a coasting period is now successfully employed in several commercial US bovine IVP businesses and has resulted in a mean of approximately 20 oocytes collected from *B. taurus* cattle once every two weeks (unpublished, courtesy of Trans Ova Genetics).

Age of the donor can also influence the number of oocytes recovered per OPU. Evidence in Holsteins suggests that a significantly higher number of oocytes can be collected in cows 6–9 years old than in cows 14–18 years old [16]. However, these were primarily cattle that were undergoing OPU/IVP due to previously existing fertility problems. Recent, unpublished data from a large commercial *in vitro* program involving reproductively healthy donors indicated little variation in recovered oocyte numbers up to age 13 and a steady decline from age 14 to 18 (unpublished, courtesy of Trans Ova Genetics). Interestingly, the developmental potential of oocytes was similar from all donor age categories in this program. In another study, young Holstein heifers aged 5–10 months tended to have significantly higher numbers of follicles, but the oocytes collected from these donors yielded fewer embryos reaching the morula and blastocyst stages [25].

A number of the factors involved in OPU/IVP cannot be covered individually without including more than one variable in the discussion. Although laparoscopic collection of oocytes was touched upon earlier in the section "Oocyte Collection from Living Cattle" and is no longer utilized in most commercial OPU/IVP programs, it is used in some programs for collection of oocytes from very young heifers. The increasingly important genomic value of young animals has led to a return to laparoscopic oocyte collection from heifers as young as two months of age [37].

There is considerable variation among donors in terms of oocyte production over time. For example, an 8-year-old Holstein donor, previously diagnosed as infertile, produced a total of 176 embryos from 167 collections by 23 different sires over a period of 167 weeks [16]. With repeated OPU collections, the mean number of oocytes decreased; however, the developmental potential of oocytes produced by OPU did not change. This clearly shows the potential for production of large numbers of embryos by OPU/IVP methods and the advantageous opportunity to use different sires weekly or even biweekly.

Oocyte Collection from Excised Ovaries

In addition to bovine oocyte retrieval by OPU, there are several situations in which oocytes are removed from excised ovaries. When cows develop terminal diseases or become crippled, ovaries can be removed via a flank laparotomy or through a vaginal incision and the oocytes recovered by follicular aspiration or slicing. Stringfellow et al. [38] reported collecting an average of 46 oocytes from 18 culled dairy cows by slicing the ovarian cortex. In a highly successful commercial program, Green and McGuirk [39] reported producing an average of 46 oocytes and 9 embryos per donor in more than 100 cases of chronically ill, injured, and senile cows.

Unpublished data from excised ovaries (author JFH) have supported successful IVP and pregnancy following transfer outcomes in many cases when ovaries were removed from crippled or "down" cows and shipped to an IVP center. In some cases, these ovaries were shipped in a warm insulated container by overnight express. Ovaries shipped at higher temperatures, including body temperature, or cooled, often did not result in the successful

production of embryos. In contrast to these results, a study in China [40] showed that a storage temperature of 15 °C for a three- to four-hour period significantly improved the quality and developmental competence of oocytes used for somatic cell nuclear transfer. The authors attributed the improvement to alleviation of stresses on the oocytes compared to those stored at 25 or 35 °C.

Oocyte Collection from Slaughterhouse-Procured Ovaries

A large source of oocytes is ovaries from cows processed in slaughterhouses. All follicles between 3 and 8 mm in diameter are aspirated and the collected oocytes used to generate IVP embryos. In a mass-production system using ovaries from the slaughterhouse, Lu and Polge [41] reported producing more than 200 000 *in vitro* derived blastocysts from approximately 700 000 oocytes in two years. Although this program is no longer in operation, it demonstrated the potential for production of IVP embryos from cattle after slaughter. Reports indicate that as many as 100 and 200 oocytes sometimes can be harvested from one pair of *B. taurus* ovaries [41]. In Japan, ovaries from individual Kobi cows are procured at the time of slaughter and sent to an IVP lab for processing. Later in the same day, carcass values of the individually identified cattle determine which oocytes are retained and processed through the entire IVP procedure. In addition to their commercial value, IVP embryos are produced in universities and labs around the world as a valuable source of material for bovine reproduction experiments [42].

Media

Once oocytes are collected, they are cultured in a series of media that support IVM, IVF, and *in vitro* development. In general, there are at least six different media utilized for the production of *in vitro* embryos. Most of these media are not commercially available and must be produced in the laboratory, though some commercial media systems exist. All of these media have common components, which are briefly reviewed here, followed by specifics about media used during specific stages of development.

Water

Pure water is the single most abundant component of all media used for all types of embryo procedures. In fact, water quality might be considered the single most important component of embryo media. What constitutes pure water and how best to produce it has been thoroughly reviewed [43].

Salts

A number of salts, such as sodium chloride and potassium phosphate, are used in the preparation of virtually all media that are utilized for IVP. These components and, in fact, all chemicals used in media preparation should be reagent quality and whenever possible they should be certified as having been "mouse embryo tested."

Energy Sources

Glucose, fructose, and/or pyruvate are often included in media as energy sources. It is well established that optimal glucose levels for bovine embryos must be low, around 0.5 mM, during early embryonic development. Once the late morula stage is reached, however, higher glucose levels are desirable, and most ET media contain approximately 2.0 mM glucose.

Macromolecules

Macromolecules include serum, bovine serum albumin (BSA), hyaluronic acid (HA), and synthetic molecules such as polyvinyl alcohol (PVA). The most important reason to include a macromolecule in IVP media is the surfactant properties of these molecules, which reduces the chances that embryos will float or stick to equipment, pipette tips, straws, and Petri dishes. More specifically, serum contains a wide range of components that are beneficial for embryo development, such as proteins, growth factors, vitamins, minerals, and energy substrates, and it is well established that serum promotes blastocyst development [44]. Serum and BSA also provide a number of physical properties, including chelation, colloidal osmotic regulation, and pH regulation. Unfortunately, the inclusion of serum during IVC has been associated with a high degree of abnormal pregnancies and offspring, often referred to as large offspring syndrome (LOS) [45–48]. (Editor's note: also see Chapter 71.)

PVA primarily is used as a surfactant in flushing and holding media that are formulated to include no components of animal origin. These media have been used on hundreds of thousands of bovine donors with highly satisfactory results, based on extensive, repeated sales and verbal expressions of satisfactions from a large number of bovine and equine ET practitioners (unpublished, courtesy of Bioniche Animal Health, Inc.).

HA is a glycosaminoglycan that is present in follicular, oviductal, and uterine fluids [49]. Embryos have been shown to have surface receptors for HA, and it is involved in the regulation of gene expression, cell proliferation, and cell differentiation. Addition of HA to bovine embryo

culture systems improves development to the blastocyst stage, increases hatching rates, and improves cryotolerance [50–53]. The traditional source of HA has been cock's combs, but it can be produced biosynthetically in a *Streptococcus equi* fermentation that does not contain any products of animal origin.

Amino Acids

The addition of all 20 essential and non-essential amino acids to embryo culture systems is almost universal. The role of certain individual amino acids and also that of all 20 combined in the development of embryos, and enhancing their viability was reviewed by Gardner [54]. When serum is absent from culture media, the inclusion of amino acids substitutes for some of the benefits that serum provides.

Antioxidants

Antioxidants reduce the formation of damaging free radicals in media. Glutathione, BSA, ascorbic acid, and catalase are frequently used as antioxidants in embryo media.

Chelators

Chelators are chemicals that bind heavy metal ions. In a perfectly pure medium, there would be no need for chelators, but there is always a chance that traces of heavy metals can enter the formulation via water or reagent salts. Transferrin, BSA, and ethylenediaminetetracetic acid (EDTA) all function as chelators.

Osmolytes

Osmolytes are organic compounds that play a role in maintaining the volume of cells and organelles within cells as well as fluid balance. When cells swell due to external osmotic pressure, membrane channels open and allow efflux of osmolytes, which carry water with them, restoring normal cell volume. Glycine is an osmolyte and the smallest of the so-called non-essential amino acids that are usually added to culture media and to some formulations of holding and cryopreservation media. The physiological basis for the osmolyte properties of glycine in embryo culture was described in detail by Steeves et al. [55].

pH and Buffers

The intracellular pH (pHi) of *in vitro* derived bovine embryos is 7.2, as measured with a pH-sensitive probe [56]. This study also demonstrated that bovine embryos in culture recovered after being subjected to an acid pH more readily than from a basic pH. In fact, pHi was decreased only to approximately 7.8 when embryos were subjected to a pH above 8. In a classic study, Kane [57] showed that the highest percentage of rabbit blastocysts that hatched during IVC was in the range of 7.2–7.4, with a precipitous decline when pH was below 7.0 or above 7.8. Most bovine IVP media are in the 7.3–7.4 range, except for fertilization media which are generally 7.5–7.6.

Phosphate buffered saline (PBS), which has been widely used for *in vivo* derived (IVD) embryo recoveries, contains phosphate as a buffer. Although phosphate is not a particularly effective buffer, the inclusion of either serum or BSA adds additional buffering capacity to PBS. IVP media, on the other hand, are normally maintained in an incubator with a 5% CO_2 atmosphere. The inclusion of bicarbonate in these media provides a stable pH due to the equilibrium that is established between the bicarbonate and the CO_2. When media based on this system are removed from the incubator, however, the pH rapidly decreases and will reach an unacceptable level of acidity within a few minutes in air. As a consequence, the media used for rinsing ova/zygotes between IVP steps and for holding them in the air prior to transfer or cryopreservation contain 3-(4-morpholino)propane sulfonic acid (MOPS) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) zwitterionic buffers.

Osmolarity

The optimum osmolarity for maintaining bovine embryos during IVP procedures is approximately 275 mM, which also is true of other species such as the rabbit, rat, and pig [58]. On a practical basis, bovine, and probably equine embryos are not adversely affected by exposure to osmolarities ranging from 250 to 300 mM for relatively short periods. Research has suggested that a total osmolarity between 250 and 270 appeared to be optimal when bovine *in vitro* derived embryos were cultured in potassium-supplemented simplex optimized medium (KSOM) [59].

An important consideration when adjusting the osmolarity of media is the issue of what molecules are added or reduced in order to raise or lower osmolarity. The most abundant salt in all media utilized in IVF procedures is NaCl, but it had been shown to be detrimental to bovine embryo development at concentrations above 95 mM [59]. Thus, the benefit of having osmolarity below 300 mM in most studies likely is due to lowered Na^+ as opposed to lowered osmolarity per se (oviduct fluid is >300 mM).

Maturation

Much of the success of embryo IVP depends on the quality of the starting material, the oocyte. Photomicrographs of the four basic categories of freshly collected oocytes are shown in Figure 89.2. Oocytes in these four categories have greatly different potentials for maturation and development into embryos, ranging from type 1 with the best potential to type IV with almost zero potential [16]. Table 89.2 demonstrates the potential for these oocytes to be fertilized, develop to blastocysts, and result in pregnancies.

An oocyte that is considered competent for IVP is referred to as "matured." Maturation involves a series of events that begin in fetal life with the initiation of meiosis. At birth, oocytes are arrested at the diplotene stage while continuing to grow and they do not become meiotically active again until puberty, when they are exposed to preovulatory surges of the gonadotropins luteinizing hormone (LH) and FSH which induce changes in gene expression as well as in morphological and physiological features of the theca, granulosa, cumulus, and oocyte compartments of the ovarian follicle. A subset of oocytes is continually recruited to proceed with meiosis during each estrous cycle, only to arrest at metaphase II, the stage at which they are ovulated. The changes that occur between the diplotene and metaphase II stages of arrest are considered maturation [61].

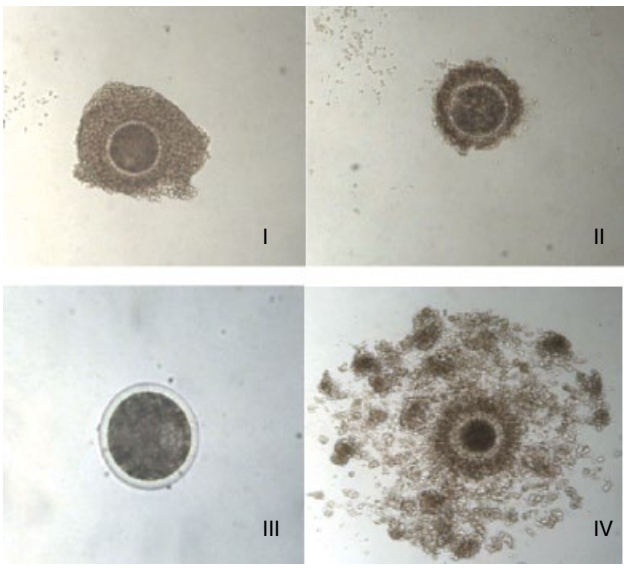


Figure 89.2 Types of bovine oocytes collected from cattle ovaries prior to IVM. I, >4 layers of cumulus; II, 1–3 layers of cumulus; III, nude oocyte with no cumulus; IV, expanded cumulus. *Source:* Reproduced with permission from [60].

IVM of immature oocytes occurs by a different mechanism than *in vivo* matured oocytes. Removal of an oocyte from the inhibitory follicular environment followed by favorable culture conditions results in spontaneous maturation without the physiological series of events that occur *in vivo*, though some morphological changes can be observed (e.g. expansion of cumulus cells) [62]. *in vitro* conditions simulate some of the physiological changes with the addition of hormones, including LH, FSH, and 17β-estradiol, which are typical of the preovulatory follicle, and growth hormones or epidermal growth factor [63].

The way in which oocytes are collected and their maturation status dictate how they must be handled after collection. Immature oocytes can be collected from unstimulated cows undergoing OPU every three to seven days, irrespective of the stage of their estrous cycle; however, it is also possible to time aspiration after ovarian stimulation and injection of LH or GnRH so that one collects mature oocytes. These *in vivo* matured oocytes do not require further maturation *in vitro*, though some period of time in maturation medium prior to fertilization may be beneficial. Oocytes aspirated from ovaries collected from cows processed in slaughterhouses are primarily from follicles 3–8 mm in diameter and the resulting cohorts of oocytes vary in their maturational status. Even so, the majority of these oocytes can achieve maturation during a 24-hour period of IVM.

While many oocytes do reach metaphase II after a maturation period, research indicates there may be an asynchrony in nuclear and cytoplasmic maturation of oocytes *in vitro*. Studies to balance this asynchrony have

Table 89.2 Potential for fertilization and embryonic development in different types of immature bovine oocytes.

Oocyte type	Cumulus	No. of oocytes	Percentage cleaved	Percentage blastocysts	Percentage hatched
I	>4 layers	571	65^{a}	29^{a}	19^{a}
II	1–3 layers	228	40^{b}	8^{b}	6^{b}
III	Nude	289	38^{b}	$<1^{c}$	$<1^{c}$
IV	Expanded	151	38^{b}	7^{b}	5^{b}

^{abc} Values within a column with different superscripts differ significantly ($P < 0.025$).

attempted to delay nuclear maturation by addition of meiotic, protein synthesis, or phosphorylation inhibitors to the maturation medium to allow more time for cytoplasmic maturation. Early indications were that this can improve maturation rates as well as embryo production rates, though results from replication experiments have been varied [64].

Other avenues of research to improve IVM and subsequent embryo development or quality involve modifying maturation media with antioxidants (e.g. cysteamine) [65], amino acids (e.g. L-carnitine) [66], hormones (e.g. thyroid hormone) [67], or maturing oocytes in a co-culture system with theca cells but no hormonal supplementation [68], to name a few. Additionally, the effects of the maturation environment on epigenetics and genomic imprinting in the IVP embryo continue to be of interest.

Fertilization

Once IVM is complete, oocytes are ready to be fertilized. This involves the co-incubation of oocytes with spermatozoa and is generally done in a four-well dish, but also can be done in a microdrop under oil. Most labs allow for 18 hours of co-incubation, even though the majority of fertilization events will be complete by 12 hours of co-incubation.

Upon ejaculation, spermatozoa are motile but not capable of fertilizing an oocyte. The changes that a spermatozoon must go through before it is capable of fertilizing an oocyte are collectively called capacitation. Even though this process was discovered in 1951 by Chang [69] and Austin [70], the molecular mechanisms of capacitation remain unclear. Capacitation generally occurs in the isthmus of the oviduct, although in some species it may begin in the cervix where cervical mucus facilitates the removal of sperm surface proteins. The challenge for those performing IVF is to mimic these conditions by providing spermatozoa with an environment that supports capacitation. Temperature is a critical factor for successful capacitation. Evidence in sheep and pigs suggests that capacitation is most efficient at body temperature rather than at 37 °C, the temperature to which many incubators are preset (reviewed in [71]). This may be associated with the physical state of membrane lipids outside of physiological conditions. In cattle, IVC is typically done at 38.5 °C, including the fertilization process.

Media have been developed to support capacitation *in vitro*. The medium in which sperm and oocytes are co-incubated is generally slightly higher in pH (7.5 vs 7.3 in the maturation and culture media, respectively). A basic medium that can be used is Tyrode's or Krebs-Ringer's solution supplemented with an energy source (glucose, lactate, and pyruvate) and albumin [71]. The primary capacitation agent added to fertilization media is heparin, which is thought to assist in the removal of seminal plasma components from the sperm surface by binding to proteins and stimulating the efflux of cholesterol and phospholipids [71, 72]. Other capacitation agents include caffeine, an inhibitor of phosphodiesterases; bicarbonate, which induces pKa-dependent changes in lipid architecture; adenosine, which interacts with membrane receptors and increases intracellular cyclic adenosine monophosphate (cAMP) and reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and nitric oxide, which activate membrane targets to trigger intracellular mechanisms including protein tyrosine phosphorylation [72].

The majority of spermatozoa used in bovine IVF are frozen–thawed. The cryopreservation process induces changes in bovine spermatozoa that resemble capacitated spermatozoa. They have elevated intracellular calcium, a net loss of plasma membrane proteins, and an increase of ROS [73]. These sperm also require a shorter exposure to and lower doses of capacitation agents than fresh spermatozoa, which are ideal for IVF. In a well-managed IVF laboratory, fertilization rates with frozen spermatozoa can average 80% or higher.

The most common method for preparing spermatozoa for fertilization is by centrifuging spermatozoa through a density gradient. This discontinuous gradient is prepared with a sperm tyrode albumin lactate pyruvate (TALP) solution mixed with Percoll® (Sigma) in different proportions. The most common gradient is a 45% Percoll mixture layered on a 90% Percoll mixture. The semen or contents of a thawed straw of semen are layered on top of the gradient and centrifuged at 800g for a time dependent on the volume of the column through which the sperm must travel. This isolates intact sperm at the bottom of the centrifuge tube. An alternative method for isolating intact sperm is a "swim up," which involves layering a medium over the contents of a thawed straw and allowing the spermatozoa to swim up into this medium, where they are aspirated off after a period of time.

Today, the use of spermatozoa that have been sorted via a laminar flow device, based on whether they carry an X or Y chromosome, is an important part of commercial bovine IVP, particularly in the dairy industry [74]. The accuracy of the semen sorting process is approximately 90%. In a number of studies, there was no difference in pregnancy rates between dairy cows receiving IVP embryos produced with sexed or traditional semen. However, a small but significant increase in pregnancy loss of IVP-sex sorted embryos compared to cows that had been artificially inseminated with traditional semen has been reported [75].

Starting in 2014, Sexing Technologies, the Navasota, Texas-based company responsible for providing a major share of sexed semen, announced a new sorting process termed SexedULTRA™ [76]. Higher pregnancy rates when this product was used in artificial insemination (AI) have been reported, but no definitive improvements in IVF are as yet available.

In some cases, frozen semen is sorted for sex after thawing and used to fertilize oocytes immediately after sorting. This is called reverse sorting and has resulted in *in vitro* blastocyst production and pregnancy rates that are comparable to rates obtained with fresh sexed semen and better than sexed semen that was frozen after sexing (unpublished, courtesy of Trans Ova Genetics) [77].

In Vitro Culture of Embryos

The IVC of bovine embryos is the last step in the IVP process and involves approximately six days of culture from the 1-cell zygote stage following IVF to a blastocyst-stage endpoint.

In the past, one of the most common media for culturing bovine embryos was variations of Tervit's [78] original synthetic oviduct medium (SOF), supplemented with amino acids and, in some cases, low levels of serum [51, 79–81]. SOF-based culture may involve one medium formulation for the duration of IVC or a two- or three-step "sequential" system in which the medium formulation changes at certain points in the culture period. Sequential systems are based on an attempt to mimic the physiological changes that *in vivo* zygotes encounter as they move down the oviducts and into the uterus during the first six or seven days of development. The volume of medium used in IVC systems also varies, with some laboratories culturing embryos in microdrops as small as 25 µl under a layer of oil, and ranging up to 500 µl in each well of a four-well dish with or without an oil layer. More sophisticated culture systems have been developed, such as microfluidic IVC [82] and single embryo time lapse culture systems, but these are not widely used at this time. Some IVC systems are conducted in an incubator atmosphere of 5% CO_2 in air; however, there is significant evidence that in the absence of co-culture, bovine embryos develop better in a 5% CO_2, 5% O_2, and 90% N_2 atmosphere [83, 84].

In the past, most commercial IVP involved the use of co-culture systems that included a monolayer of somatic cells. Cell types that have been used in the successful co-culture of bovine embryos include primary cells from oviducts (bovine or porcine), mouse oviductal ampullae, cumulus, granulosa, and uterus, as well as established cell lines such as buffalo rat liver (BRL), and Vero cells. These cells have been combined in co-culture systems with a number of different media, including TCM-199, Menezo's B2, and Ham's F-10.

Co-culture systems are not widely used today, as most laboratories have largely pursued investigating IVP systems based on "defined" media formulations. Defined media are those in which all of the components are known and, as a consequence, products such as serum and BSA are not included. It should theoretically be possible for a defined system to be exactly replicated in any laboratory at any time. Also, the use of defined systems is essential for understanding and optimizing the culture requirements of pre-implantation embryos. However, only moderately successful rates of blastocyst production have been reported with the use of chemically defined, protein-free media [85, 86]. Some very comprehensive reviews have been written on this subject [87–90].

Commercial ET/IVP businesses are primarily concerned with efficiently and predictably producing as many viable embryos as possible. Consequently, BSA (semidefined media) and, in some cases, moderate levels of serum (non-defined media) are frequently added to various media, including IVC media. Addition of high concentrations of FCS (10% or greater) has been associated with lipid accumulation in the embryo, which decreases cryotolerance, and with the production of large calves, though lower concentrations (<5% or less) of FCS may mitigate these effects while still enhancing embryo production. Timing of supplementation with fetal bovine serum (FBS) or preparation of the FBS may also influence embryo production results [91], though for practical as well as animal welfare reasons alternatives to FBS are being researched [92, 93]. In addition, non-defined systems may involve co-culture in which any of a number of different somatic cells is included in the system. These systems can be difficult to reproduce because of variations among batches of commercially available cells or a lack of "immortality" when some cell types are cultured through multiple generations in IVP laboratories.

Continual efforts to improve IVC and results post transfer involve supplementing media with components found *in vivo*. There is evidence that a variety of factors can improve embryo development as well as establishment and maintenance of pregnancy. For example, insulin-like growth factor-1, colony stimulating factor 2, and hyaluron are found in the female reproductive tract and when added to IVC media are reported to enhance embryo survival post transfer (reviewed in [94]). There is an extensive body of literature on supplements and/or additives that may improve embryo development rates *in vitro*.

Numerous qualitative and quantitative differences between *in vivo* and *in vitro* produced embryos have been

described in detail for cattle and other species [16]. There is evidence that IVP embryos have fewer cells and lower proportions of inner cell mass (ICM) cells to trophoblast cells than *in vivo* embryos at the same stage of development and this variation can be related to different culture systems [95, 96]. However, total cell counts do not necessarily reflect the overall integrity and viability of preimplantation IVP embryos. Van Soom et al. [97] used differential staining to show that although embryos produced in Menezo's B2 medium had higher cell counts than embryos of the same age produced in TCM-199, the B2 embryos had a larger range of variation in the number of ICM cells. The additional cells in the embryos produced in B2 were mainly trophoblast. Including serum can also increase cell numbers, but there is evidence that this may decrease the embryo's ability to survive cryopreservation [98]. Thus, one should not choose an IVC medium strictly on the basis of increasing cell numbers in embryos.

In addition to differences in cell numbers, the rate of embryonic development varies among IVC media [99–101]. It is clear that within a given IVC medium, faster developing embryos frequently are of higher quality based on cell number [102], end-stage of development [103], and pregnancy rate after transfer [33].

A study that utilized fluorescence in-situ hybridization showed that a high proportion (72%) of bovine embryos cultured in a Menezo's B2 co-culture system were mixoploid (being composed of polyploid and diploid cells), compared to 25% of blastocysts developed *in vivo* [104]. Of the total number of *in vitro* embryos, 17% contained more than 10% polyploid cells. In contrast, all of the *in vivo* embryos contained less than 10% polyploidy cells. The authors of this study offered a hypothetical link between the polyploidy in *in vitro* embryos and the possibility of polyploidy placental cells developing from them, leading to overactive placentas that in turn are responsible for large calves (see section "Pregnancies and Offspring").

A summary and illustration of early embryo development in the IVP process compared to *in vivo* embryo development is provided in Figure 89.3.

Cryopreservation of IVP Embryos

Cryopreservation of bovine embryos is routinely done in research and commercial IVF laboratories around the world. Traditional slow-freeze cryopreservation has been used extensively with IVP embryos, with pregnancy rates up to 50–55% in large-scale trials [33, 105]. Pregnancy rates with frozen–thawed IVP embryos have been lower, and thus globally most IVP embryos are transferred fresh (73.2%) compared to only 39.9% of IVD embryos in 2018 [106]. As the number of IVP embryos generated annually continues to rise and exceed the number of *in vivo* produced embryos (1 029 400 IVP compared to 469 967 IVD in 2018) [106], improving cryopreservation of bovine IVP embryos will continue to be an important area of research.

A rather unique finding was reported in a paper from Japan in which bovine IVP embryos were successfully held for seven days at 4 °C in a HEPES-buffered medium [107]. This approach eliminated the need for cryopreservation and the authors opined that embryos could be held and transported for up to seven days with a high pregnancy rate following transfer into recipients.

The most common protocol for slow-freeze cryopreservation of bovine oocytes involves cooling and holding the

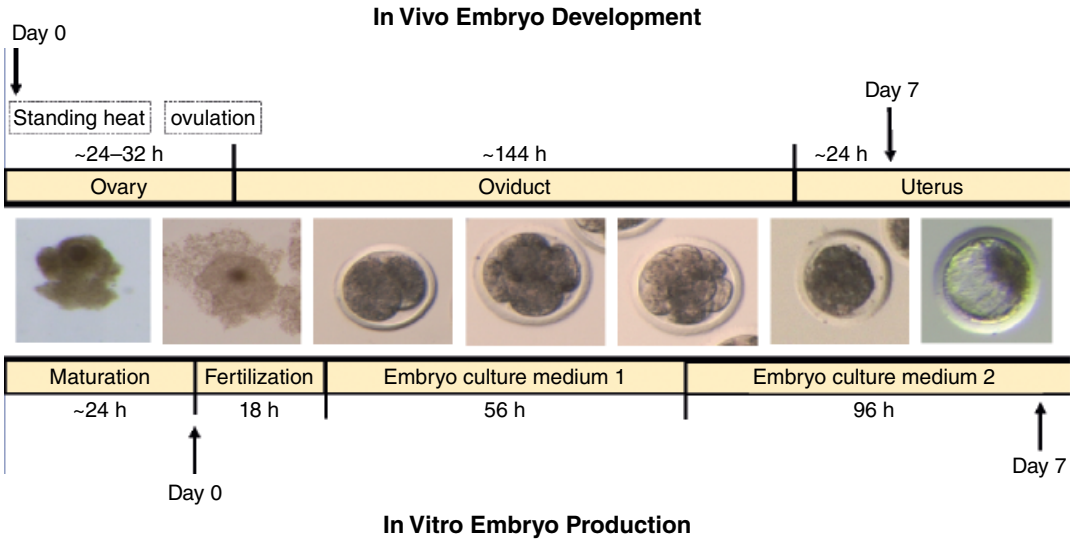


Figure 89.3 Comparison of early embryo development *in vitro* to *in vivo*.

embryo at −6 °C, during which time the straw containing the embryo is seeded, followed by a decrease to −32 °C at 0.3–0.6 °C per minute and plunging the straw into liquid nitrogen. Bovine embryos are typically frozen in a solution containing 1.5 M ethylene glycol and 0.1–0.5 M sucrose. The use of ethylene glycol as the cryoprotectant allows one to transfer the embryo to a recipient directly from the straw, whereas other cryoprotectants such as glycerol must be removed via dilution of the freezing medium prior to transfer [108]. Other cryoprotectants that have been used to freeze bovine embryos include dimethyl sulfoxide (DMSO), propandiol, methanol, various sugars, and combinations of these [109].

There are several factors that influence embryo survival post thaw, including stage of development. Morulae and blastocysts survive the freeze–thaw process better than earlier-stage embryos, but even the day of blastocyst development can impact post-thaw survival. Pregnancy rate following transfer of IVF blastocysts frozen on day 7 was 42%, but dropped to 20% for blastocysts frozen on day 8, with a higher incidence of early abortions and dystocias [33]. The quality of the embryos also impacts post-thaw results. Pregnancy rates for frozen–thawed bovine embryos by grade were reported by Hasler et al. [33]: grade 1 – 63%, grade 2 – 57%, grade 3 – 44%, and grade 4 – 36%.

There has been extensive research conducted on the effects of cooling and warming rates on embryos of a variety of species. In one study, bovine embryos were cooled at 0.3, 0.6, 0.9, 1.2, or 1.5 °C/min and warmed either rapidly in a 35 °C water bath (warming rate >1000 °C/min) or slowly in 25–28 °C air (<250 °C/min) [110]. The best treatment was cooling at 0.3 °C/min with rapid warming; 42.1% of the morulae developed into hatching blastocysts. These results indicate that the survival of IVP bovine morulae and blastocysts is improved by very slow cooling, but slow warming was deleterious to morulae and blastocysts even when coupled with very slow cooling.

Vitrification is an alternative to slow-freeze cryopreservation that involves ultra-rapid cooling, resulting in a glass-like state rather than ice formation. Since its introduction as an effective embryo cryopreservation method in 1985 [111], vitrification has been used primarily for research. Limitations of vitrification include toxicity from exposure to high concentrations of cryoprotectants, damage caused by osmotic shock, the need for highly skilled technicians, and the lack of a convenient direct transfer method. Researchers have attempted to improve vitrification of bovine embryos by manipulating culture or vitrification media [112, 113], delipidation of embryos, and/or altering plasma membrane composition [114, 115], as well as reducing free water in embryos by either predehydration or reduction of the blastocoele cavity prior to cryopreservation [116]. Despite these challenges, vitrification has proven more effective for embryos sensitive to cryopreservation, such as IVP embryos [117]. Another advantage of vitrification is that it does not require expensive cell freezers used for slow-freezing procedures and the equilibration and subsequent cryopreservation take less time per embryo. In addition, studies have shown that slow-freeze cryopreservation may induce changes in expression levels of genes involved in development in the preimplantation bovine embryo when compared to vitrified embryos [118], though further research is needed. Vitrification combined with an in-straw dilution would provide an alternative to slow-freeze cryopreservation of embryos produced *in vitro* or *in vivo*.

IVP embryos have been produced from vitrified bovine oocytes, but embryo production rates remain low [119]. Most protocols involve vitrification of mature oocytes (in metaphase II) as this has been more successful than vitrification of immature oocytes; however, damage to the spindle and microtubule network as well as the cytoplasmic membranes remains a problem [115].

Transfer of IVP Embryos

In most cases, IVP embryos are transferred in the same manner as IVD embryos. Once removed from the incubator, IVP embryos are usually transferred immediately, whereas IVD embryos can be held without loss of viability at room temperature for up to 24 hours prior to transfer.

Pregnancy rates of IVP embryos are consistently lower than IVD embryos. Significantly lower pregnancy rates were achieved, in order, by IVD frozen, fresh IVP day-7, fresh IVP day-8, frozen IVP day-7, and (lowest) frozen IVP day-8 embryos [33]. With regard to stage of development independent of age and grade, the transfer of large numbers of IVD embryos showed that early blastocysts (EB) and mid-blastocysts (MB) resulted in higher pregnancy rates than morulae (M), expanded blastocysts (XB), or hatched blastocysts (HB) [120]. Transfer of grade 1 day-7 embryos at the EB, MB, or XB stages results in higher pregnancy rates than transfer of corresponding day-8 embryos.

Synchrony of recipient cows to embryo development is an important factor for the establishment of pregnancy. For IVP embryos, day 0 is considered to be the day of IVF, while in cattle, including ET recipients, day 0 commences when the cow is in standing estrus. With regard to ET, zero synchrony traditionally refers to the transfer of an embryo into a recipient of the same day, e.g. transfer of a day-7 embryo into a recipient that was in estrus 7 days earlier. Minus 1-day synchrony is the transfer of an embryo to a recipient that was in estrus 1 day earlier than the age of the embryo,

e.g. a day-7 embryo transferred into a cow that was in estrus 6 days prior. While this is the convention, it is misleading because ovulation in the recipient occurs approximately 12 hours after the end of estrus which lasts on average 18 hours (day 0 begins at the onset of standing estrus), while fertilization *in vitro* occurs within hours. Consequently, if an IVP embryo is designated as day 7, it is actually one day older than a day-7 IVD embryo [33, 121]. Pregnancy rates reflect this asynchrony. When day-7 embryos were transferred into day-6 recipients, pregnancy rates were 50.3%, compared to 55% for day-7 and 58% for day-8 recipients. Further support is provided by data based on more than 10 000 transfers from Trans Ova Genetics showing pregnancy rates of 42, 50, and 49% when day-6, -7, and -8 recipients, respectively, were used for day-7 IVP embryos (personal communication). To avoid decreased pregnancy rates, the use of day-6 recipients should be avoided whenever possible.

Commercial production of bovine embryos via IVP is now successful in a number of laboratories around the world. BOVITEQ Inc. (Quebec, Canada) [122] and Trans Ova Genetics (Sioux Center, IA, USA) [123] and Em Tran, Inc. (Elizabethtown, PA, USA) [33] either have been in the past or are currently operating commercial ET companies that have successfully produced OPU/IVP derived pregnancies from infertile donors. In addition, Trans Ova Genetics, Sexing Technologies, and Ova Tech in the USA and BOVITEQ in Canada are currently producing large numbers of IVP embryos from healthy cattle.

The trend in the international production of IVP and IVD embryos on a biyearly basis between 2000 and 2018 is shown in Figure 89.4. It is clear that *in vivo* production, involving the number of embryos produced by superovulation and collection via "flushing," is decreasing, whereas there has been a rather dramatic, steady increase in the numbers of IVP embryos. Initially, more IVP embryos were produced in Brazil than in any other country. One company, *In Vitro* Brazil, reported the transfer of more than 200 000 IVP embryos in 2012. In 2007 it was estimated that superovulation/flushing represented only 9% of the ET work in Brazil, while OPU/IVP provided 91% of the embryos (unpublished, courtesy of *In Vitro* Brazil). Although such a large difference in how embryos are produced is not true in every country, the increased emphasis on OPU/IVP versus superovulation/multiple ovulation embryo transfer (MOET) is evident worldwide, as shown in Figure 89.4.

There has been especially dramatic growth of OPU/IVP in the USA in recent years, along with a decline in the traditional MOET. The number of actual collections for these two technologies is shown in Figure 89.5.

In the Netherlands, IVP embryos have been produced from oocytes obtained by OPU from young, reproductively healthy donors and transferred on-farm around the country [124]. In Japan, the production of IVF-derived embryos from slaughterhouse ovaries has been commercialized, and in some cases very large numbers of embryos have been produced. Kuwayama et al. [125] described the production of more than 150 000 frozen IVP embryos in a one-year period from more than 21 000 ovaries of Japanese Black cattle.

Today, much of the OPU/IVP industry is driven by the increased sophistication and accuracy of genomic analysis of individual animals, especially in breeds in which a great deal of information is available from the contribution of individual bulls. Genomics has had a profound effect on the selection of individual animals that enter OPU/IVP programs. This trend and the reasons for it have been

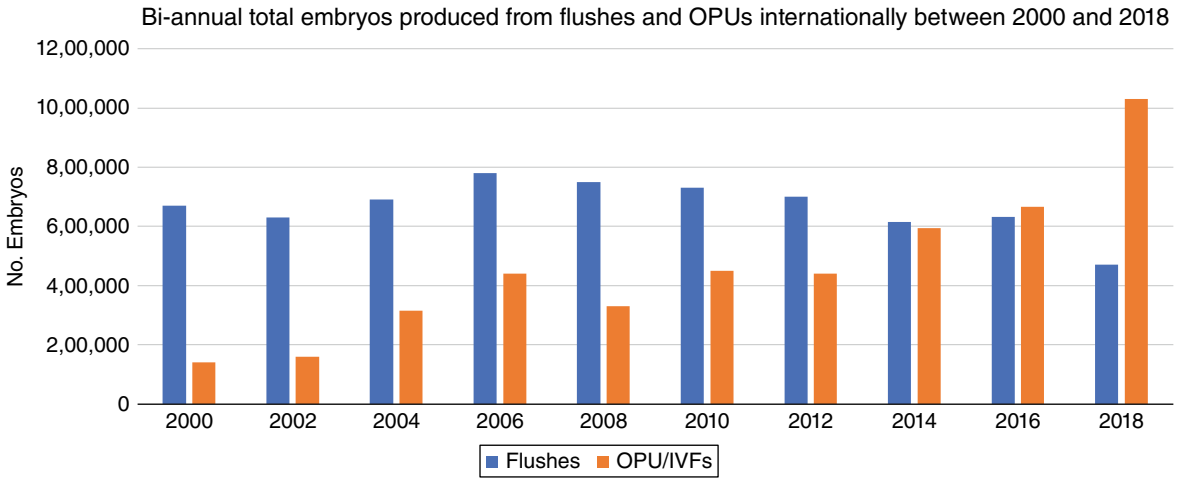


Figure 89.4 Comparison of biannual number of embryos produced from flushes and OPUs (combined beef and dairy) internationally between 2000 and 2018. *Source:* Based on Compliments of the International Embryo Technology Society.

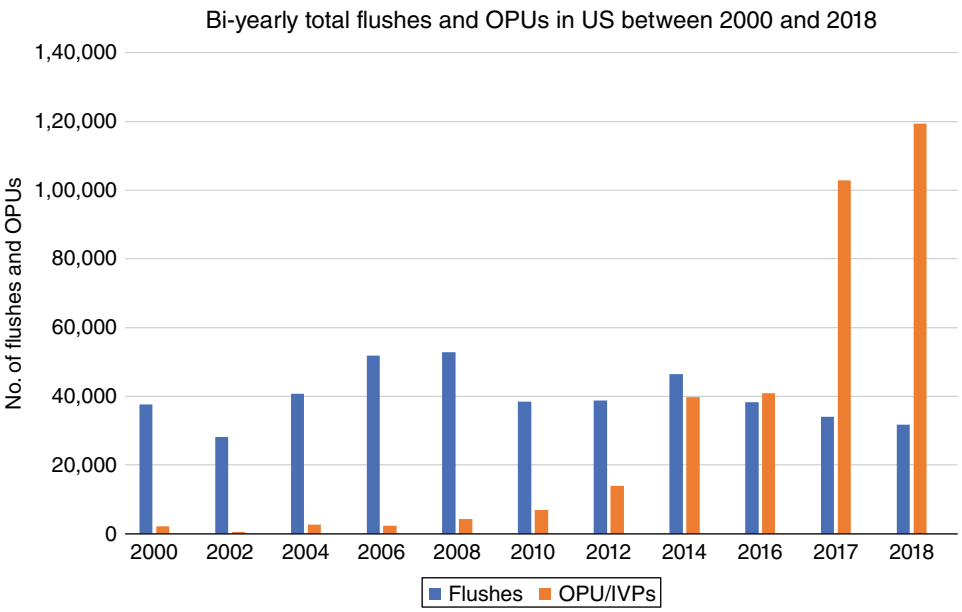


Figure 89.5 Comparison of biannual number of flushes (producing *in vivo* embryos) and OPUs (combined beef and dairy) in the USA between 2000 and 2018. *Source:* Based on Compliments of the American Embryo Transfer Association.

covered in a number of reviews including [126–128]. The importance of genomic value has probably been the main reason that the overall application of superovulation/MOET has decreased, while OPU/IVP has increased in dairy donors since 2000 [129]. (Editor's note: see Chapter 76.)

Pregnancies and Offspring

Problems with pregnancies resulting from bovine IVP embryos have been described by a number of authors [16, 47, 80, 124, 130]. One of the more obvious problems is larger-than-normal calves at birth, referred to as LOS. Some of the problems associated with this syndrome are shown in Table 89.3.

A number of reports indicate that the average birth weights of IVF calves are heavier than those of AI calves [124, 131, 132]. Furthermore, Farin and Farin [130] showed that seven-month fetuses derived from IVC embryos were heavier than those from *in vivo* embryos. Other studies indicate that serum, co-culture, IVM–IVF, and uterine environment are all capable of influencing fetal development in sheep. These studies have primarily been done with sheep because the high maintenance costs and longer gestation period make the bovine a more expensive model for studying the effects of *in vitro* procedures on subsequent offspring. Nevertheless, the findings that lambs from *in vitro* cultured embryos exposed to serum can be up to 20% heavier than control embryos could be of significant consequence to the cattle industry [133]. Longer gestations have also been noted but are not correlated with the weight of the offspring [133–135].

Table 89.3 Aberrant characteristics associated with the LOS syndrome of IVP bovine pregnancies and calves.

Large calves
Increased gestation length
Decreased intensity of labor
Increased rate of abortions
Increased rate of congenital malformations
Increased rate of perinatal mortality
Increased rate of hydroallantois in recipients

Because serum has been implicated as a cause of higher birth weights of *in vitro* derived lambs (reviewed in [45]), its use in bovine IVC systems has received a good deal of attention. Deletion of serum during the first four days of co-culture failed to decrease the incidence of problems with IVP derived pregnancies and calves [46], whereas Agca et al. [136] showed that birth weights and gestation lengths were normal for a relatively small number of calves resulting from embryos cultured in serum-free CR1aa [137]. Another study comparing pregnancies resulting from co-cultured embryos versus those produced in SOF containing BSA showed that the use of SOF resulted in lower birth weights and easier calvings [80]. Unfortunately, comprehensive data regarding late-term pregnancies, incidence of

dystocias, and calf normalcy are not available from the large commercial *in vitro* programs currently in business.

Higher-than-normal abortion rates, ranging from 8 to 47%, during the first two trimesters of pregnancy were reported for *in vitro* derived bovine pregnancies [33, 138–140]. In contrast, abortion rates of 5.3% from 2 to 7 months [141], 4.7% from 2 to 6 months [120], and 4% from 40 to 250 days [142] for bovine *in vivo* ET pregnancies were reported.

Perinatal and neonatal losses have also been reported as higher in recipients carrying *in vitro* derived fetuses compared to AI or *in vivo* embryo pregnancies [33, 124, 143, 144]. There was no difference in the incidence of calf loss at birth between calves produced in the TCM-199 and B2 co-culture systems at Em Tran. However, the overall loss at birth of the IVF calves was 14.9% (205/1376), which is higher than the 9% loss reported for *in vivo* ET [141].

Increased incidence of hydrallantois was diagnosed among pregnant Holstein recipients carrying IVP derived fetuses (1%; 34125). A frequency of 1% is substantially higher than the cited frequency of 1 per 7500 calvings reported in non-IVP pregnancies [145].

Finally, although the data were not quantified, it was reported that labor often was not clearly pronounced in the Holstein heifers used as recipients for IVP embryos [16]. As a result, these recipients often were not observed in labor, and calvings frequently went unassisted, resulting in increased perinatal losses. However, when calves were delivered by scheduled cesarean section, the survival rate was close to 98%.

A number of reports show that male bovine IVP embryos grow more rapidly than female embryos during the first seven to eight days in IVP [146–149]. Bovine IVP embryos are usually transferred on day 7 or 8 and the most advanced embryos are usually selected because they tend to be more viable [33]. The percentage of male calves born following transfer of IVP embryos has been reported as 60% [138], 62% [140], and 53.4% [16]. The 53.4% was based on more than 2000 IVP calves and fetuses and differed significantly ($P < 0.001$) from 50% and also from the 51.1% males reported for 1751 calves produced from transferred IVD embryos [141] and the 50.5% of 24 000 Holstein AI-produced calves that were males [150].

Summary

IVF has come a long way since 1959 when the first IVF mammal was produced, and is now a growing component of the bovine ET industry. Media systems continue to be improved to more closely mimic the *in vivo* environment, resulting in better quality embryos. This, coupled with the introduction of sexed semen to the IVP process, and the availability of very comprehensive genomic values, has enhanced the value of IVP embryos, particularly in the dairy industry. Additionally, cryopreservation techniques have been greatly improved for IVP embryos, making on-farm transfers very convenient and facilitating the storage and shipment of these embryos.

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90

Cloning by Somatic Cell Nuclear Transfer

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Introduction and Brief History

In 1997 Dr. Ian Wilmut and coworkers [1] startled the world by announcing the birth of Dolly, the first ever clone (i.e. genetic replica) of an adult mammal (Figure 90.1). Because doing so was thought to be biologically impossible, the announcement of Dolly's birth reverberated around the world, capturing the attention of scientists and conjuring up outlandish visions from the lay public. Fascination with Dolly, continuing even today, stems from her unexpected origin. Dolly originated from a mammary cell derived from a six-year-old adult ewe. The mammary cell's nucleus was transferred into an oocyte whose nuclear DNA had been previously removed (i.e. enucleated). The brief electrical pulses used to achieve that end were all that was needed to initiate embryonic development, highlighting the important fact that a spermatozoon is not needed for this type of "asexual" reproduction.

Ability to clone by nuclear transfer was not novel to Dolly. In fact, numerous clones were produced pre-Dolly using totipotent or pluripotent cell types comprising the early embryo. The first successful production of embryonic derived clones by nuclear transfer was in amphibians in 1952 [2]. Thirty-one years later McGrath and Solter [3] announced embryonic cell cloning of mice. Soon thereafter came embryonic cloning of sheep [4], cattle [5], rabbits [6], and pigs [7]. Global failures using other differentiated, highly specialized cell types gave credibility to the long and widely held dogma that it was biologically impossible to clone adult mammals by nuclear transfer. An initial hint that this dogma would not withstand the test of time came with the reporting of the births of Megan and Morag in 1996 by Dr. Wilmut's group (Dr. Keith Campbell as lead author) [8]. Unlike all the other clones that had been born up to that time, these cloned sheep were obtained after performing nuclear transfer using a cultured cell line that was embryonic in origin but had been passaged up to 6–13 times in the laboratory (i.e. number of times the cell line was subcultured to produce a larger number of cells from the initial number harvested). Because cells were serum starved before nuclear transfer, success at producing cloned offspring was attributed to the use of quiescent-induced cells (i.e. non-proliferating and induced into G_0 of the cell cycle). Subsequent cloning successes using established cell lines from mammary (i.e. Dolly), fetal, and embryo cells after inducing cells into a quiescent state (Table 90.1) reinforced this notion.

Although it was later shown that inducing cells into quiescence before nuclear transfer was not required, Dolly's birth continues to serve as a reminder that the impossible is possible assuming a willingness to persevere while thinking "outside the box." The list of adult animals cloned using different cell types and cultured cell lines for nuclear transfer now includes, but is not limited to sheep, cattle, mouse, goat, pig, gaur, mouflon, rabbit, cat, horse, rat, African wild cat, sand cat, mule, banteng, deer, dog, ferret, wolf, buffalo, camel, and Spanish ibex (Figure 90.2). Realizing what can be done in one species is likely possible in others, the list of species that will be cloned will continue to grow. In 2018, macaque monkeys were added to the list. Use of cumulus cells to derive nuclear transfer clones from adult females yielded two live births that were short-lived [9]. Because clones may be constructed using a variety of different body cell types, cloning by nuclear transfer is appropriately termed somatic cell nuclear transfer (SCNT).

Procedures Involved

The SCNT procedure is remarkably similar in the numerous species that have been cloned to date, highlighting the relevance of the procedural steps depicted in Figure 90.3.

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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Companion website: www.wiley.com/go/hopper/bovine



Figure 90.1 Dolly, a lamb derived from the mammary gland of a Finn Dorset ewe, with the Scottish Blackface ewe which was the recipient. *Source:* [1], with permission from Nature Publishing Group. Image kindly provided by the Roslin Institute, University of Edinburgh.

Although any/all are important to obtain viable offspring, success is ultimately dependent on reprogramming somatic nucleus to allow for proper embryo development. The cell type of choice is an oocyte arrested at metaphase II (MII). An oocyte at this stage of meiotic arrest has within it the majority, if not all, of the key critical components to direct early embryo development.

Collection of somatic cells from the animal to be cloned (i.e. somatic cell donor) is an important first step in SCNT. In cattle and other species, adult animals have been cloned using a variety of different diploid ($2n$) cell types including, but not exclusively, fibroblasts obtained from various sources, granulosa cells from antral follicles, and cumulus cells, along with cells originating from mammary tissue, muscle, oviduct, uterus, and other sources. From a practical viewpoint, the relative ease of obtaining skin-derived fibroblasts using a simple biopsy instrument makes this cell type a logical choice to use for cloning adult males and females. Ovarian/granulosa cells are also easily obtained from mature females using the same ultrasound-guided transvaginal aspiration equipment used for ovum pick-up (OPU) in cattle. Regardless of origin, cells obtained from somatic cell donors may be utilized soon after collection, or more often cultured for some time in the laboratory before being frozen for later use. Inducing quiescence in somatic cells using serum starvation or other approaches is not required to obtain cloned offspring. Before SCNT is to be performed, cells are dispersed into a single cell suspension. A trypsin-based solution works well with most somatic cell types. In cases where outcomes are less than expected for a certain somatic cell donor (i.e. embryo development or pregnancy outcomes less than expected after performing SCNT) effort to establish a new cell line, rather than switching cell type, may be beneficial.

When SCNT is to be performed, oocytes must be collected. In cattle, pigs, sheep, goats, and horses, abattoir-derived ovaries provide an abundant and affordable supply of oocytes. In cases where it is important to control the genetic and/or maternal background of the females providing oocytes to reprogram somatic nucleus, OPU procedure provides a means for collecting smaller/limited numbers of oocytes from live animals. There is no doubt that risks exist when oocytes and other biological materials from non-verified sources are used for *in vitro* production of embryos [10, 11]. However, being mindful of the importance of biosecurity along with effort to use source-verified materials, ensure proper oocyte and embryo handling, and disease testing whenever possible and relevant are important steps to minimize inherent risks.

After collection and extensive washing, cumulus oocyte complexes must be matured *in vitro*. In most maturation

Table 90.1 Development of cloned sheep embryos constructed with three different cell types.

Cell type	No. of fused couplets (%)	No. of morulae/ blastocysts (%)	No. of pregnancies/ no. of recipients (%)	No. of live lambs
Mammary epithelium	277 (63.8)	29 (11.7)	1/13 (7.7)	1 (0.36%)
Fetal fibroblast	172 (84.7)	47 (37.9)	5/16 (31.3)	3 (1.74%)
Embryo derived	385 (82.8)	126 (54.5)	15/32 (46.9)	4 (1.04%)

Source: From [1], © 1997, Springer Nature.



Figure 90.2 Different animals cloned by SCNT; camel, sand cat, Spanish ibex, water buffalo, macaque monkey, and wolf are not shown. *Source:* When possible, images were obtained from relevant journal articles after obtaining publisher permission (i.e. Nature Publishing Group and Elsevier). Images of a cloned gaur and banteng and permission for use were provided by Dr. Robert Lanza of Advanced Cell Technology. Texas A&M College of Veterinary Medicine and Biomedical Sciences provided images and permission to include cloned cat and deer. Dr. Martha Gomez at the Audubon Center for Research of Endangered Species provided image and permission to include an African wildcat.

systems, the majority of oocytes will have matured and progressed to MII by 18–20 hours after placement into maturation medium. Ability to remove associated cumulus with ease and visually identify those that have extruded a polar body provides a greater level of control for selecting MII oocytes.

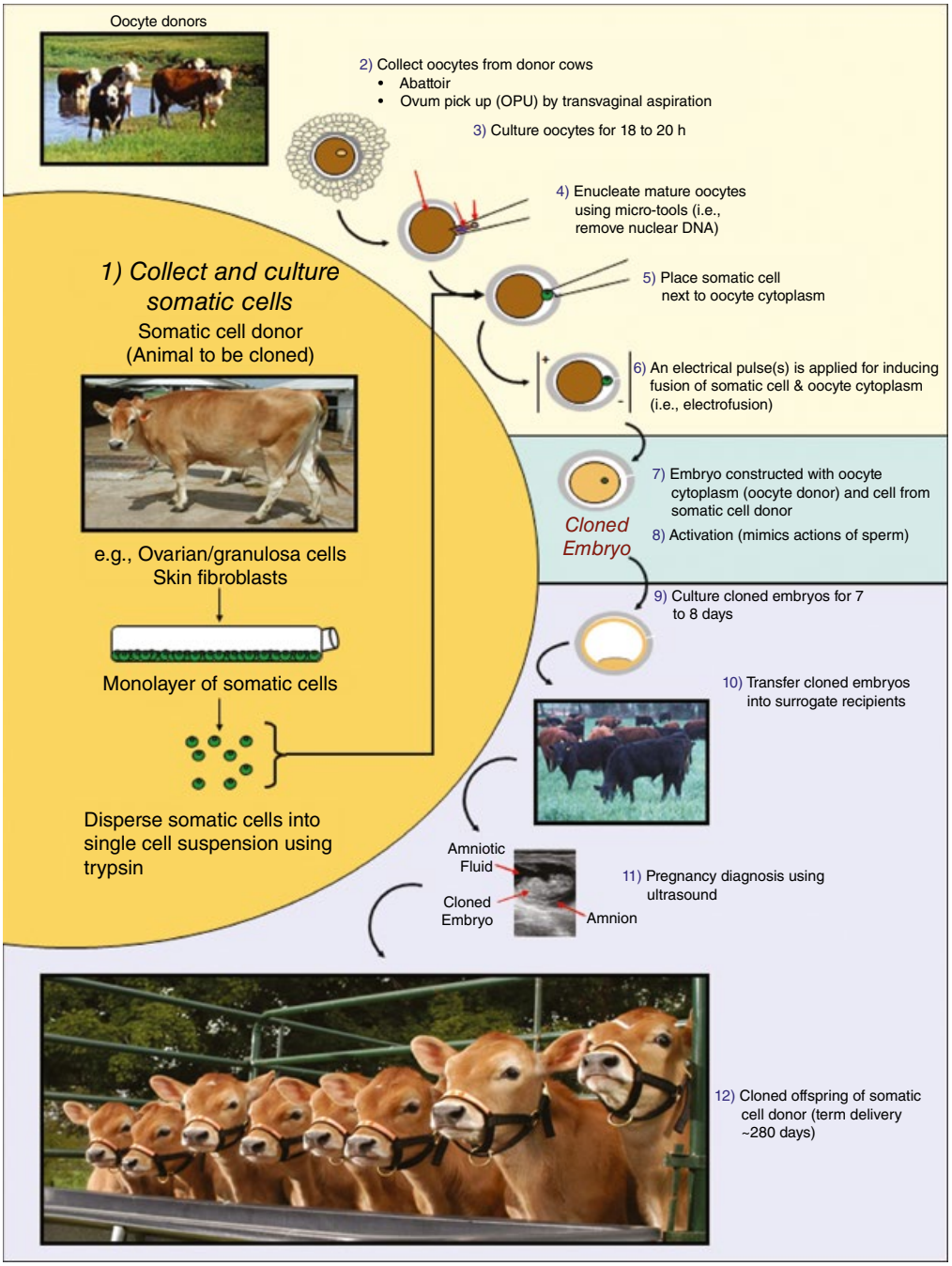


Figure 90.3 Different SCNT steps for cloning adult animals. Although the schematic depicts efforts to clone a cow, SCNT procedures are similar across species (1). The first step involves collection of cells from the animal to be cloned (somatic cell donor) (2). Oocytes are collected from other cows (3). Oocytes may be matured *in vitro* (4). DNA is removed from an oocyte using microtools (5). A somatic cell is placed beside the bag of oocyte cytoplasm (6). Exposure of the oocyte cytoplasm and somatic cell to an electrical current will induce the majority to fuse (7), effectively constructing a 1-cell cloned embryo (8). Electrical induction of fusion of the oocyte cytoplasm with somatic cell may be sufficient to activate the cloned embryo to begin development (9). Cloned embryos may be cultured in the laboratory for a defined period of time (10). Thereafter, embryos must be transferred to surrogate recipients for continued development (11). Ultrasound is useful for determination and monitoring of resulting pregnancies (12). A few of the cloned embryos will develop to term, resulting in the birth of live offspring.

The next and perhaps more labor-intensive step of the SCNT procedure involves the removal of maternal (nuclear) DNA from MII stage oocytes using microtools (Figure 90.3). Addition of a microfilament inhibitor (i.e. cytochalasin B) to the holding medium relaxes the cytoplasm, allowing mechanical removal via aspiration of 5–15% of the oocyte's cytoplasm containing the maternal (nuclear) DNA. Doing so is important to minimize the incidence of lysis that

would otherwise occur without the addition of a microfilament inhibitor. Addition of a DNA-specific fluorescent dye (i.e. Hoechst stain) to the holding medium allows verification that the oocyte's nuclear DNA was successfully removed. Taking effort to restrict/limit exposure of ultraviolet light to the microtool containing maternal DNA that was removed is important to avoid issues/harm related to exposing mitochondrial DNA, organelles, and other essential cytoplasmic components.

The next challenge of the SCNT procedure is transferring the nucleus from a cell obtained from the somatic cell donor (i.e. animal to be cloned) into the "bag" of oocyte cytoplasm that resulted after enucleation of MII chromatin. In most species, mice being an exception, this is achieved by electrical-induced fusion. To this end an intact somatic cell is mechanically inserted into the perivitelline space (available space between the zona pellucida and the enucleated bag of oocyte cytoplasm) using microtools. The resulting "couplet" (bag of enucleated oocyte cytoplasm and somatic cell) is aligned between two electrodes and briefly pulsed with an electrical current. In cattle, 2.2 kV/cm for 40 μs induces >70% of couplets to fuse. Applying an electrical current in this manner induces pore formation in the respective membranes of the two cell types. Electrofusion as a means of transferring the somatic nucleus into the bag of oocyte cytoplasm depends on continuing direct contact between the somatic cell and the oocyte cytoplasm. Depending on the extent to which membranes intermingle, fusion may occur. Within minutes of introducing the somatic nucleus into the bag of oocyte cytoplasm, the nuclear membrane breaks down and the chromatin condenses. Successful fusion of this couplet effectively constructs the equivalent of a 1-cell embryo.

In some cases, the electrical pulses utilized to transfer the somatic nucleus into the enucleated oocyte cytoplasm is also sufficient to "activate" components in the oocyte cytoplasm which is critical to initiate embryo development. Some laboratories prefer the use of different chemical combinations to achieve this end (i.e. calcium ionophore and/or different kinase inhibitors). Regardless of how one chooses to "jump start"/initiate embryo development, the ultimate challenge of activation protocols is to mimic the developmentally important processes occurring after fertilization. Note the deliberate use of the word "mimic" because the spermatozoon is *not* required for this type of asexual reproduction. After activation and depending on the species, cloned embryos may be transferred into ligated oviducts (preventing entry into the uterus of temporary recipients; this is what was done for Dolly) or the uterus (pigs), or cultured in the incubator for a period of time required for development to the compact morula or blastocyst stage (cattle). Cloned bovine embryos develop to the blastocyst stage at an equivalent rate to those undergoing *in vitro* fertilization (Table 90.2).

Because of the incredible effort to produce an SCNT embryo and pregnancy, outcomes are less when *in vitro* derived embryos are frozen, and morula-/blastocyst-stage SCNT embryos should be transferred into surrogate recipients without delay. Embryo transfer utilizes the same approach as is done when transferring other *in vitro* or *in vivo* derived embryos. In cattle, effort should be taken to transfer embryos before or during the early stages of blastocoele expansion, otherwise the expanding blastocoele that is extruding through the holes in the zona pellucida resulting after insertion of microtools to enucleate the oocyte or insert somatic cell into perivitelline space may be problematic (Figure 90.4). Extensive extrusion resulting from the expanding blastocoele through a hole in the zona pellucida has been likened to a "figure 8"

Table 90.2 Developmental potential of SCNT cow clones (constructed with ovarian/granulosa cells and skin fibroblasts) or *in vitro* produced embryos using *in vitro* fertilization.

Embryos	No.	No. cleaved (%)	No. of day 6 and 7 morulae and blastocyst-stage embryos (%)
Clones	686	ND	207 (30.2)
in vitro produced	863	705 (81.7)	235 (27.2)

Source: Adapted from [12].
ND, Not determined.

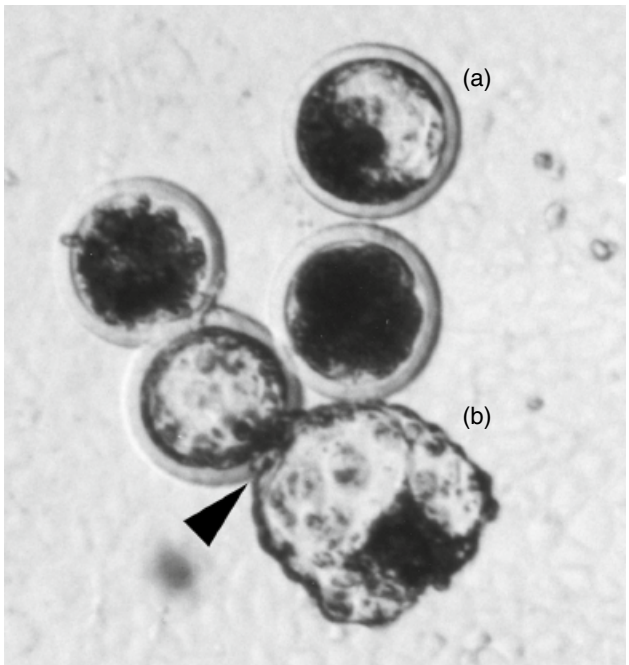


Figure 90.4 Bovine cloned blastocysts (a) before expansion and (b) expanding blastocyst where much of the blastocoele is extruding through the hole in the zona pellucida (arrowhead) giving a "figure 8" appearance.

(Figure 90.4). The potential for extrusions of this type to become pinched/broken off during or after transfer into the uterus may explain why on occasion cloned pregnancies result in twins after the transfer of individual cloned embryos. Depending on the level of difficulty during transfer, potential for damage increases when transferring embryos in the process of expanding. Because pregnancy outcomes after the transfer of one or two embryos do not differ, effort to transfer a single embryo is helpful to minimize complications associated with twinning. To avoid issues with the aberrant hatching of SCNT embryos, earlier stage embryos should be transferred (i.e. compact morula- or early blastocyst-stage embryos). When doing so, embryos should be stage-matched with the uterus (i.e. compact morulae would be transferred to day-6 recipients, not day 7 or 8). Depending on the cell line, it is reasonable to expect day-28 pregnancy rates in cattle approaching 50% after the transfer of single SCNT embryos, as indicated by the presence of an embryo proper with heartbeat (Table 90.3). A limited number of cloned embryos resulting in a confirmed pregnancy will progress to term and result in the delivery of live offspring.

Applications

Success in cloning sheep, cattle, pigs, and goats confirms the usefulness of SCNT for clonal expansion of agriculturally important food-producing animals. Additional efforts of producers to cross their best clonal lines to create new genetic combinations are imperative for making genetic improvements while maximizing genetic diversity. It is worth restating that SCNT clones are merely genetic replicas of existing individuals. Relevant for the few clones that could eventually enter the food chain, compositions of their meat and milk products are indistinguishable from those that are derived from conventionally bred animals [14, 15]. The US Food and Drug Administration (FDA) confirmed that there are no unique risks with meat or milk products from clones or their progeny [16], affirming safety for consumption of food-derived products [15].

The usefulness of SCNT to restore endangered or even extinct species is closer to being realized. Initial reports described efforts to preserve the last surviving cow of the Enderby Island cattle breed [17] and the cloning of a wild ox (guar bull) which was on the verge of extinction [18]. In these and others instances, oocytes within a given species are not available for SCNT. Reprogramming a somatic nucleus from an endangered species is accomplished by transferring to an enucleated oocyte obtained from a different species. Outcomes of interspecies SCNT are less than those expected for same species SCNT [19]. Depending on species-specific differences, incompatibility of mitochondrial and nuclear genome along with other things beyond the scope of this chapter may continue to be problematic.

Use of SCNT is commercially available for individuals seeking to clone companion animals. "To clone or not to clone" depends on expected outcomes. In instances where genetic background of a given animal is not known or

Table 90.3 Comparison of SCNT pregnancy losses in the cow related to MHC homozygous and heterozygous SCNT cell lines.

Cell line	Year	MHC type[a]	No. of embryos transferred	No. of pregnant recipients (%)			
				Day 28[b]	Day 90[c]	Day 200[c]	Term[c]
MHC class 1 homozygous. SCNT cell lines							
UT3388	2001	AH68/AH68	48	29 (60.4)	21	21	13
UT3388	2002	AH68/AH68	21	6 (28.5)	5	5	4
UT4585	2002	AH67/AH67	39	10 (25.6)	8	6	6
		Total	108	45 (41.6)	34	32	23
MHC class 1 heterozygous SCNT cell lines							
UT4381	2001	AH12/AH68	48	18 (37.5)	0	0	0
UT4472	2002	AH12/AH68	5	3 (60)	1	1	1
		Total	53	21 (39.6)	1	1	1

Source: From [13], © 2004, Elsevier.

[a] MHC class I haplotypes (AH) carried by the SCNT donor cell lines were determined by MHC class I microarray typing and genomic MHC class I sequencing.

[b] Day-28 pregnancy rates following transfer of a single SCNT embryo to each recipient were not significantly different for the two groups ($P = 0.80$).

[c] Pregnancy losses for class I homozygous and heterozygous SCNT cell lines were compared for three time periods: days 28–90, days 28–200, and day 28 to term. For all three time periods, pregnancy loss was significantly different at $P < 0.001$ (chi-square test).

limited, SCNT may provide an opportunity to produce a genetic replica of an existing individual. However, in instances where intent is to use technology to "replace" a deceased companion, outcomes of SCNT will be less than expected. For instance, identical twins (i.e. nature's truest form of clone) are genetically identical but phenotypically different because of environmental influences.

Cloning by SCNT for the purpose of producing genetic replicas of existing individuals is collectively referred to as "reproductive" cloning. Because SCNT could be used for cloning humans, the moral and ethical issues of its use have been, and will continue to be, debated by various governments worldwide. While the majority would agree that cloning humans for reproductive purposes should be banned, the usefulness of the technology for therapeutic purposes remains contentious. Therapeutic cloning refers to the use of SCNT to reprogram patient-derived somatic cells to provide a source of patient-derived stem cells that could be used for the regeneration of tissues afflicted by a disease or injury. Use of patient-derived stem cells would obviate concerns related to tissue or cell rejection. Contentious aspects of SCNT for this purpose relate to destroying resultant embryo to harvest stem cells for patient/donor use.

Ability of SCNT to utilize somatic nucleus to construct an embryo also allows for cell-mediated transgenesis [20]. Its use for producing transgenic animals (a.k.a. genetically modified organisms) is dependent on inserting a foreign gene of interest into the somatic cell nuclear genome *before* SCNT is performed. Use of a transgenic cell line in SCNT effectively increases the efficiency of producing transgenic farm animals to 100%. Mindful of this and that somatic cells can be easily cultured in the laboratory, genetically modified, and tested before nuclear transfer, makes SCNT the preferred approach for producing transgenic farm animals [20]. The ability to produce transgenic farm animals to serve as bioreactors for the production of pharmaceuticals or possibly serving as organ donors for the human population has created a new form of "pharming" with unlimited potential benefit for human health. Transgenic animals also provide a means in agricultural and biomedical disciplines to study genes involved in a variety of biological and physiological systems.

Challenges Associated

A major limitation of SCNT to clone agriculturally important food-producing animals and other species continues to be the inefficiency in producing live offspring. Dolly was one of 277 cloned embryos that developed to term (0.3% efficiency) [1]. "Millie," a clone of an adult Jersey cow produced in the laboratory of the authors Edwards and Schrick, was just one offspring that resulted after 95 attempts (1.1% efficiency). The US Food and Drug Administration (FDA) conducted a detailed assessment of the potential hazards and risks to highlight the magnitude of inefficiencies that continue to be problematic in food-producing animals such as cattle, sheep, pigs, and goats [15]. Data provided by numerous academic and private laboratories around the world emphasize that, regardless of species or somatic cell type, deaths of cloned embryos and fetuses occur throughout pregnancy. Complications occurring later in pregnancy pose significant risks to the surrogate recipient. Moreover, an unusually high proportion of cloned offspring that are born are larger than normal and die soon after birth.

Development of cloned bovine embryos during the first 29–32 days parallels development of embryos produced after *in vitro* maturation, fertilization, and culture (IVMFC) to the blastocyst stage (Figure 90.5 and Table 90.3). However, similarities beyond this time period end abruptly. Depending on the cell line, embryonic deaths (defined by the absence of a heartbeat and detachment of placental membranes [24]) may occur in up to 100% of cloned pregnancies between about 30 and 90 days of pregnancy (Table 90.3 and Figure 90.6). Without ill effects on the dam, pregnancy losses in cattle of this magnitude during this time period are significantly higher than those expected after breeding by natural service (2–10%) [25] or after development using IVMFC procedures (16%) [21, 22]. Detailed efforts examining placentas from cloned embryos between days 40 and 50 of gestation showed that a high percentage were hypoplastic or partially developed with rudimentary cotyledons [24] (Figure 90.7). Placental insufficiency likely explains much of the fetal losses occurring during this time period. Although the basis for placental insufficiency is not entirely understood, immune-mediated rejection related to inappropriate expression of trophoblast major histocompatibility complex (MHC) class I antigens may be problematic [26]. While other efforts did not provide evidence to support abnormal expression of MHC class I on the trophoblast of cloned fetuses [27], it is interesting to note that the two cell lines used extensively in our laboratory (Edwards/Schrick) and shown to produce a substantial number of successful pregnancies were both derived from MHC homozygous cows [13] (Table 90.3). In contrast, the cell lines resulting in a high degree of fetal losses were from MHC heterozygous cows, suggesting some degree of incompatibility.

The incidence of spontaneous abortions increases during the second trimester of cloned pregnancies. Complete macroscopic and histopathologic examinations of aborted fetuses reveal few abnormalities. However, placentas are oftentimes grossly abnormal with a marked reduction in

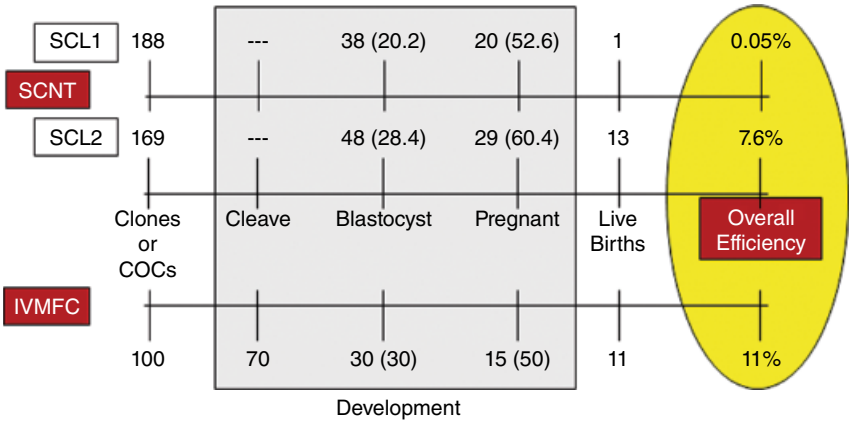


Figure 90.5 Comparison of overall efficiency of SCNT using different somatic cell lines (SCL1 vs SCL2) with IVMFC procedure to produce offspring. Numbers presented for SCL1 and SCL2 represent actual numbers obtained using two different cell lines. Numbers of cumulus–oocyte complexes (COCs) presented for IVMFC were derived from our own experience and volumes of data presented by Dr. John Hasler in published literature [21–23]. Shaded areas highlight time periods with similar development for SCNT and IVMFC embryos (i.e. up to ~29 days in gestation with pregnancy rates approximating 50%).

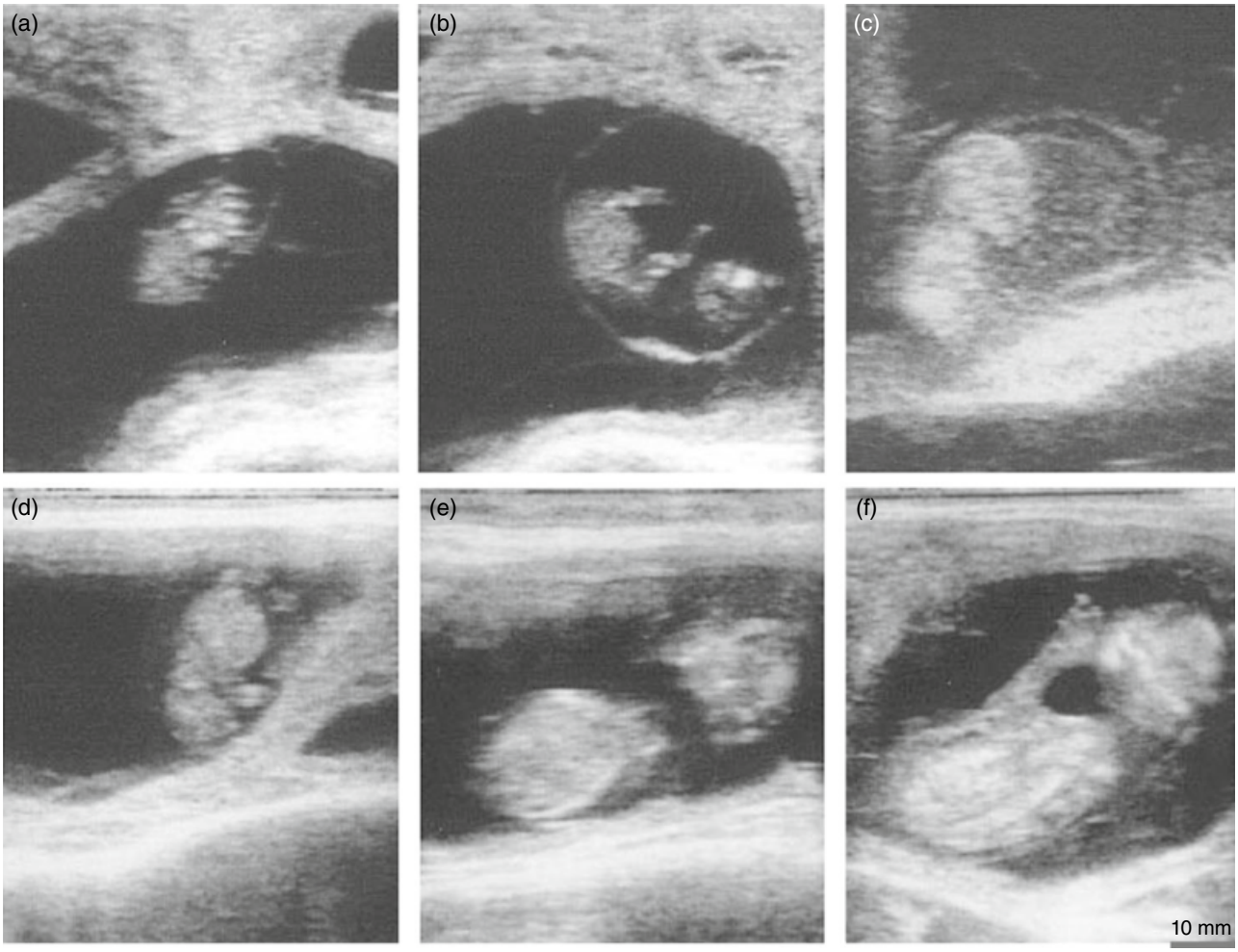


Figure 90.6 Comparison of development of non-viable (a–c) and viable (d–f) SCNT fetuses using transrectal ultrasonography at days 45 (a, d), 55 (b, e), and 65 (c, f). The non-viable fetus was alive at day 45 (heartbeat detected) and dead at day 55 (no heartbeat). At day 65, increased echogenicity of amniotic fluid was apparent as the fetus degenerated. Crown–rump lengths (cm) for the non-viable and viable fetuses at each stage were, respectively, 3 vs 2.8, 3.7 vs 5.7, and 3.7 vs 7.5. *Source:* [24], with permission from Wiley-Blackwell. Image kindly provided by Dr. Jonathan Hill at the University of Queensland.

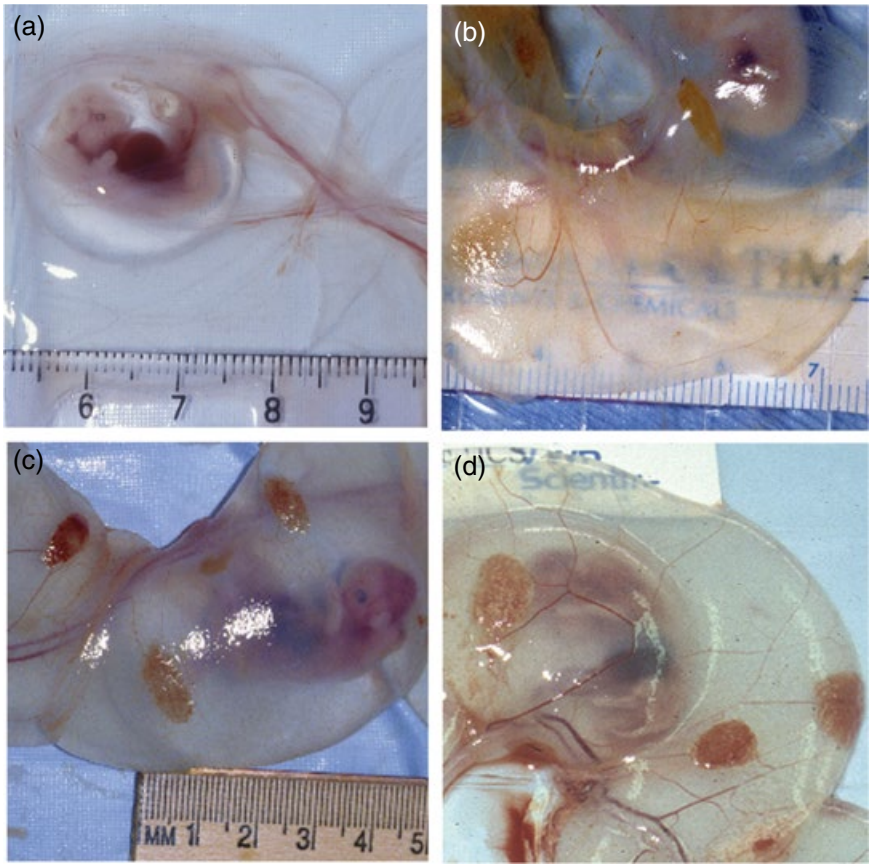


Figure 90.7 Each observed SCNT placenta could be grouped as hypoplastic (a, day 40), partially developed with a reduced number of rudimentary cotyledons (b, day 45), or essentially normal (c, day 50). A control placenta is included for reference (d, day 53, placenta from an IVMFC derived fetus). *Source:* [24], with permission from Wiley-Blackwell. Image kindly provided by Dr. Jonathan Hill at the University of Queensland.

fetal cotyledons (occasionally fewer than 20 compared with the expected 70–120) [15] (Figure 90.8). Fetal membranes are generally thickened and edematous.

Pregnancy losses during the third trimester, especially between 200 and 265 days (term is 280 days), are more challenging. Many of the fetal losses occurring during this time relate to an increased incidence of the dropsical condition hydrops. Excessive accumulation of fluid may occur in different parts of the placenta (hydrallantois and/or hydramnios) [28] and/or fetus (hydrops fetalis). Incidence of hydrops as high as 86% has been reported with SCNT pregnancies with varying levels of severity [15]. In our experience, 58% of established pregnancies developed hydrops; 37% of the hydropic pregnancies were severe enough to warrant euthanasia of the surrogate recipient. Incidence of hydropic SCNT pregnancies is much higher than what may be expected in pregnancies resulting from conventional breeding (1: 7500) [29] or from IVMFC procedures (1: 158) [23].

In moderate to severe cases of hydrops, the abdomen becomes noticeably distended from the excessive accumulation of fluids (Figure 90.9a). Uterine rupture and other



Figure 90.8 SCNT fetus aborted during second trimester. Placenta was thickened and edematous with about 14 fetal cotyledons.

harmful complications to the surrogate recipient may be problematic in rapidly developing cases, warranting immediate euthanasia [28]. Attempts to "rescue" the fetus by emergency cesarean section often prove futile as the fetus is often already dead. Even if delivered early enough, neonatal care is inadequate to sustain the life of those neonates delivered at the time when most cases of hydrops are diagnosed. Marked reductions in numbers of placentomes of varying sizes, along with marked hypertrophy of many cotyledons, are evident with cloned placentas. Often there is an increased incidence of adventitial placentation and severe edema in the intercotyledonary placental membranes (Figure 90.9d). Most fetal abnormalities are consistent with consequences related to hydrops and placental insufficiency. Fetal anasarca with generalized edema and marked edema of the umbilicus is usually present (Figure 90.9e). Amniotic squames and meconium are generally present in the lungs of late-term fetuses, indicating some degree of stress in utero before death. Because of the poor prognosis for the fetus and possible harm to the surrogate recipient, termination of a hydropic pregnancy is most often warranted after initial diagnosis. Some of the cattle producers interviewed for the FDA risk assessment [15] indicated that they began monitoring surrogate recipients closely for developing signs of hydrops as early as 150 days into gestation.

While clones may die immediately or soon after birth (within about 48 hours), survival rates up to 50–70% are obtainable. Survival is related to birthweight to some extent (Figure 90.10). In the sample data provided in Figure 90.10, the majority of Jersey cloned calves surviving (L, live) weighed about 39 kg or less at birth. Jersey clones

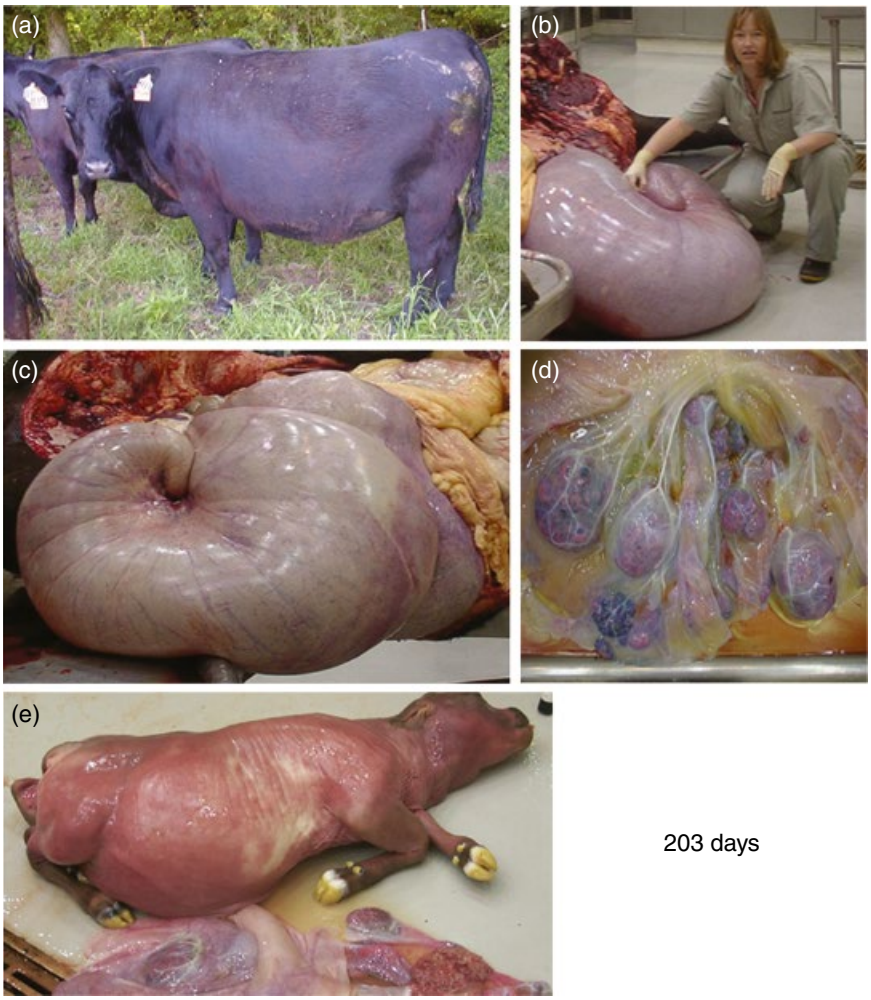


Figure 90.9 (a) Hydropic surrogate recipient at 203 days of gestation. (b) After euthanasia; ovary is located on the tip of the first uterine horn (indicated by individual's hand). (c) Clearer image of hydropic uterus; the fetus is located in the uterine horn behind the first one. (d) An abnormal cloned placenta with variations in size of placentomes (50–1 kg) and edematous fetal membranes. (e) A Jersey cloned fetus weighing 30 kg with an enlarged umbilicus. *Source:* Image in (d) reproduced from [13], with permission from John Wiley & Sons.

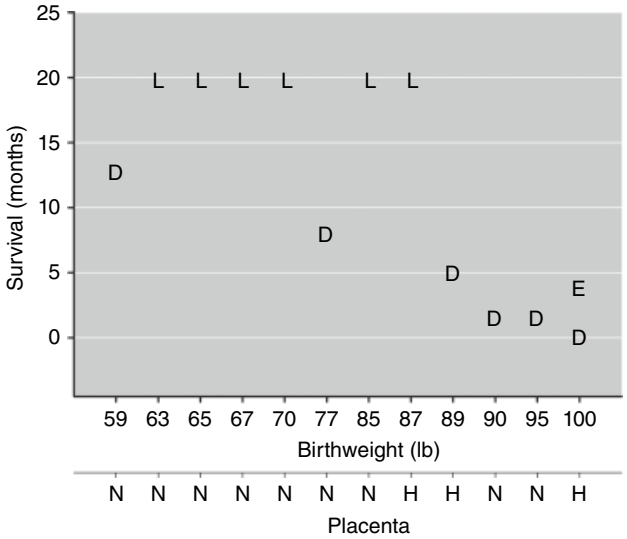


Figure 90.10 Representative image depicting survival for SCNT clones of Jersey cows (L, live; D, dead; E, euthanasia) considering birthweight and placenta (H, hydropic; N, no obvious signs of hydrops).

weighing 40–45 kg died at birth, and at times thereafter. Of the few cloned embryos that develop to term, calves are typically larger at birth, with some exhibiting clinical signs associated with "dummy calf" syndrome. Reported incidence of cloned calves exhibiting large/abnormal offspring syndrome varies considerably, ranging from 8 to 100% [15]. In our experience and others [15], large calves are slow to stand, have difficulty with regulating body temperature, have a weak sucking reflex, and their umbilicus is often enlarged and edematous with a patent urachus. Some have deformities in limbs related to tendon contracture, have disproportionate or immature organ development, and may be more susceptible to infections. Calves exhibiting clinical signs of large/abnormal offspring syndrome are intensively monitored and may be provided with therapies to treat a whole plethora of complications, including but not limited to lung dysmaturity, pulmonary hypertension, respiratory distress, hypoxia, hypothermia, hypoglycemia, metabolic acidosis, enlarged umbilical veins and arteries, and/or the development of sepsis in either the umbilical structures or lungs. Few survive, despite extraordinary efforts to intervene.

Complications in cloned calves may not be evident for several months, with the majority of deaths occurring by six months of age [15]. In those exceeding six months of age, surviving clones may appear "normal" and "healthy" until some unexpected pathology presents itself. We had three clones die at 7.5, 8, and 9 months of age, while two others died at 1 and 2.5 years of age. Although different pathologies were noted, several died or were euthanized after periodic episodes of scours unresponsive to treatment. A few but documented instances of sudden death in clones without any ill effects in the non-clones housed in the same environment makes for good discussion whether death was attributable to the animal being a clone or merely a victim of its circumstances.

Fortunately, most of the healthy-appearing clones from otherwise normal pregnancies exhibit normal growth and development. To date, numerous clones have been bred and have given birth. Dolly gave birth to six lambs (Bonnie was the first, born in 1998, twins followed the next year, with triplets the year after that; Figure 90.11). Unfortunately, Dolly was diagnosed with Jaagsiekte sheep retrovirus (JSRV) as were other sheep at the Roslin Institute. She was euthanized in 2003 after developing viral-related lung tumors according to the Roslin Institute.

Like cattle, much of the SCNT losses occurring in other species are attributable to early embryonic and fetal losses. Fortunately, complications related to hydrops and large/abnormal offspring syndrome are not problematic in goats or the majority of other species cloned to date. In pigs, litter sizes ranging from zero to eight are possible



Figure 90.11 Dolly with Bonnie, her first-born lamb after being mated to a small Welsh Mountain ram. *Source:* Image and permission to reproduce were provided by the Roslin Institute, University of Edinburgh.

after the transfer of more than 100 one-cell cloned embryos into the uterus of surrogate recipients [15]. While a few abnormalities have been noted, most pig clones are smaller, not larger, at birth when compared to those derived from natural mating or IVMFC procedures [15]. Success with cloning horses using SCNT was realized and numerous clones using adult somatic cells have been produced. Although the incidence of embryonic and fetal losses parallel those occurring in cattle (75–100% loss may be incurred depending on cell line), pregnancies and offspring in the available literature are without complications related to hydrops and large/abnormal offspring syndrome [30, 31]. For the most part, placentas appear "normal" at term, with some foals having an enlarged edematous umbilical cord.

Although SCNT in the dog is remarkably similar to what has been done in cattle and other species (Figure 90.12), inability to mature appreciable numbers of oocytes *in vitro* warrants use of *in vivo* matured oocytes [33]. Aside from dogs being monoestrus, the fact that oocytes undergo maturation in the oviducts after ovulation, which may occur over a time period of 48–72 hours, poses a unique challenge related to controlling stage of maturation utilized for SCNT. With success after transferring cloned embryos to the oviducts, much of the inefficiency related to SCNT in dogs has been related to early embryonic and fetal losses. Placental issues may be problematic with cloning of dogs. As observed in cattle, heavier placentas were associated with lower survival rates [34]. Different pathologies have been noted in non-surviving cloned fetuses and offspring, including increased muscle mass [35].

Potential Factors Contributing to the Inefficiency of Cloning by SCNT

Mindful of the physical insult inherent in the SCNT procedure and the complexity of factors within the oocyte's cytoplasm that are "expected" to reprogram a somatic nucleus, it is truly remarkable that any clones develop into healthy offspring. Some of the procedural aspects related to the extensive micro-manipulations required to construct cloned embryos using oocytes and somatic cells are not ideal (see Figure 90.3). Removal of nuclear DNA from the oocyte typically involves the physical removal of 5–15% or more of the cytoplasm while exposing it to an otherwise "noxious" chemical. Verification of this process may expose the cytoplasm to ultraviolet light, which has been shown to alter membrane integrity, increase methionine uptake, and alter protein synthesis and mitochondrial activity [36]. To transfer the somatic cell nucleus into the bag of oocyte cytoplasm generally involves electrical-induced fusion or microinjection. No matter how careful, some degree of membrane lysis occurs with either procedure. Furthermore, a variety of protocols have been described for activating cloned embryos, with the majority involving exposure to otherwise noxious chemicals. It is also important to note that SCNT typically involves the transfer of a somatic cell nucleus into an ooplast (enucleated oocyte) obtained from a nondescript female not from the somatic cell donor. This results in embryos with nuclear DNA from the somatic nucleus but mitochondrial DNA (mtDNA) from the "foreign," non-autologous oocyte cytoplasm. Resulting embryo is not a "true" clone like identical twins; rather it is chimeric at the mitochondrial level (i.e. mtDNA heteroplasmy).

Moreover, culture of cloned embryos from *in vitro* matured oocytes before transfer into surrogate recipients is not without ill effects. Like SCNT, complications inherent to the production of embryos using IVMFC procedures include alterations in gene expression in early embryos and fetuses [37], increased abortion rates (~16%) [22], abnormal placentas, increased incidence of hydrallantois (~1%) [21], heavier fetuses, dystocia, large/abnormal offspring syndrome, and mortality rates (~15%) [21–23, 38–41], albeit at a much lower rate (see Chapter 71). The specific SCNT factors exacerbating problems inherent to IVMFC procedures are poorly understood. However, stark differences in the ability of certain cell lines to produce live offspring after nuclear transfer, approaching values derived from IVMFC procedures (see Figure 90.5), implicate improper or incomplete reprogramming of the somatic nucleus by the oocyte cytoplasm. To this end, genomic methylation status in cloned blastocysts often resembles that observed in somatic cells, which would explain the aberrant expression of developmentally important genes observed in cloned embryos and fetuses [42, 43].

Summary

Cloning by SCNT defies basic principles in developmental biology. Eventual improvements related to increasing the number and survival rates of cloned embryos, fetuses, and offspring are imperative for fully realizing SCNT applications for animal agriculture and human medicine. Because of the complexity involved with the construction of an embryo, using nothing more than a somatic nucleus and the cytoplasm of an oocyte, it is likely that many more years of effort will be required to understand the process well enough to improve outcomes.

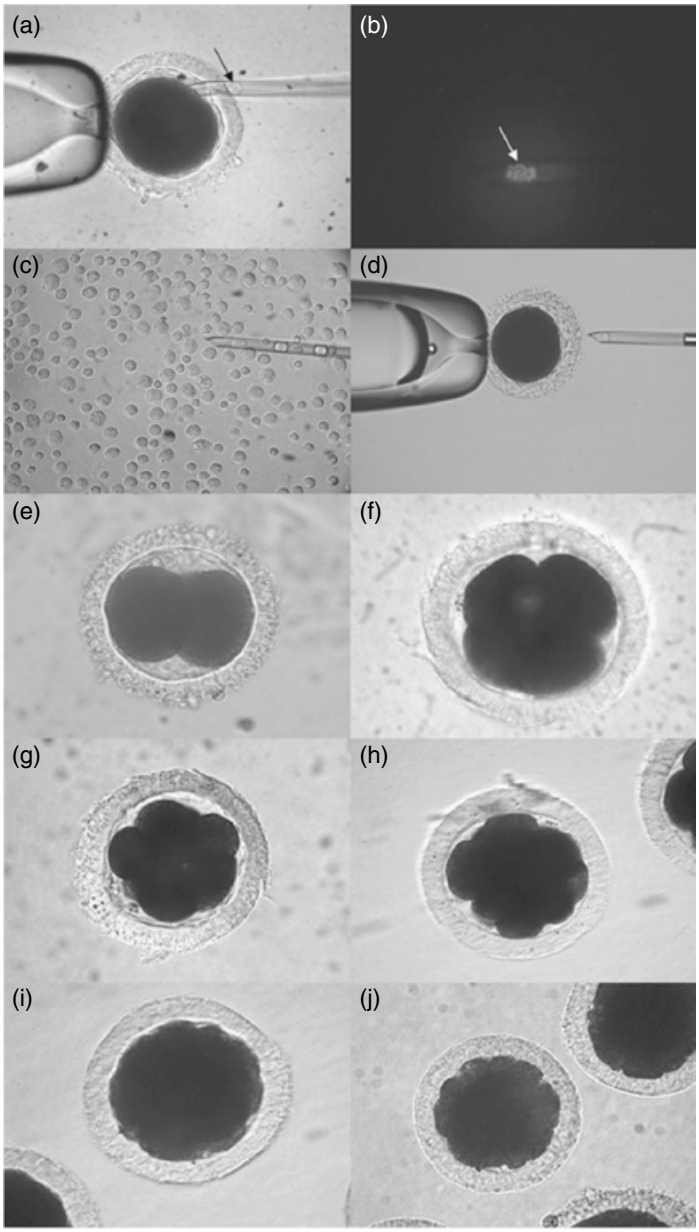


Figure 90.12 Representative image depicting SCNT procedure and *in vitro* embryo development for cloned dogs. The first polar body (a, arrow) and oocyte nucleus (b, arrow) are removed in *in vivo* matured oocytes using an aspiration pipette. Enucleation may be confirmed by nuclear staining (b, arrow). Somatic cells are aspirated into a micropipette (c) and placed into the perivitelline space of an enucleated oocyte (d). Fused couplets are activated and cultured for seven days. Cloned cleavage stage embryos: 2-cell (e), 4-cell (f), 8-cell (g), 16-cell (h), 32-cell or morula (i, j). *Source:* Reproduced from [32], with permission from Elsevier.

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Application of CRISPR-Cas9 Technology in Bovine Reproduction

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Introduction

Genome manipulation was first made possible in the 1970s when *Escherichia coli* was first manipulated to produce somatostatin, a therapeutic protein, by fusing this gene to the *E. coli* beta-galactosidase gene on a plasmid and transforming it into *E. coli* [1]. Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology is now a unique tool that can precisely change an organism's DNA. Precision genome editors are new genome editing tools that enable one to rewrite genes, making targeted changes to an organism's DNA by deleting, inserting, or modifying sections of DNA sequences with unprecedented specificity. Genome editing is currently unraveling its benefits in wide areas of science and medicine, although there are ethical, social, and legal challenges associated with its application. Advances in gene editing technologies from transgenesis and gene silencing to precision gene editing tools (zinc-finger nuclease [ZFN], transcription activator-like effector nuclease [TALEN], and CRISPR) put us at the dawn of a new revolution in life sciences.

Basics of the Genome

A genome consisting of DNA (RNA in RNA viruses) is the blueprint of life and heredity, and the instructions needed for an organism to grow, develop, survive, and reproduce are written in the genome. Mammalian body consists of trillions of cells which are divided into somatic cells and gametes. Somatic (autosomal) cells have a diploid number of chromosomes, whereas gametes comprise a haploid number of chromosomes. In the event of fertilization, two gametes, one from the dam and one from the sire, unite to form a zygote. A zygote's genome is a combination of a male and a female gamete DNA, and initially quiescent but contains all the genetic information necessary to form a new individual. The mammalian genome is comprised of billions of nucleotide base pairs, and a sequence of nucleotides constitutes a gene that encodes the synthesis of a functional gene product, either RNA or protein. A set of genes is responsible for a genotypic trait and the genotypic trait interacts with the environment to produce a specific observable phenotypic characteristic. Numerous studies have been carried out to elucidate gene function; however, elucidation of genes and their function is far from our understanding because genes are differentially expressed in a temporal and spatial manner. Modern genome editing technologies take a leap toward endogenous genome modification with the ability to rapidly and economically introduce sequence-specific alterations into the genomes of a broad spectrum of cell types and organisms, which gives rise to several opportunities to increase production and reproduction efficiency, to produce disease-resistant animals, and to generate climate-tolerant animals in livestock species.

History of CRISPR Technology

The underlying molecular mechanism of the CRISPR-Cas9 system was highlighted by a group of researchers at the University of California-Berkley and the University of Vienna in 2012 [2]. From the fact that in bacteria and archaea, CRISPR-associated systems provide adaptive immunity against viruses and plasmids by using CRISPR RNAs to guide silencing of invading nucleic acids of viruses [3–9], the researchers explained that this system can be used in prokaryotic cells to produce programmable precise double-strand breaks at any specific DNA site by

Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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combining an RNA guide with an endonuclease protein, CRISPR-associated (Cas)9 nuclease. Simultaneously, the investigators filed a patent for the methods and applications of this RNA-directed site-specific DNA modification. Concurrently, another group of researchers from the Broad Institute of MIT and Harvard University demonstrated that the *Streptococcus pyogenes* CRISPR system can be heterologously reconstituted in mammalian cells, including in several human cell lines, to facilitate highly efficient genome editing [10]. A patent application was also filed from the Broad Institute of MIT for invention of mammalian genome editing using CRISPR-Cas9 technology. Legal battles around these patents from these two groups of researchers have attracted media attention and made the CRISPR fantasy in recent years [11–13].

The simple, albeit elegant, machinery of the CRISPR-Cas system has become the technology of choice in various biotechnological applications including genome editing, gene therapy, and elucidation of gene function. With the accuracy and flaws in techniques, and ethical and social implications from the public, CRISPR-Cas9 technology and its applications are being progressed rapidly. The CRISPR-Cas9 system has two crucial molecules, Cas9 and guide RNA (gRNA), that can generate an accurate change in DNA. The endonuclease protein Cas9 acts as a pair of "molecular scissors" that can sever two strands of DNA at a specific location in the genome in order to delete or insert fragments of DNA. The gRNA is a small piece of predesigned RNA sequence (about 20 nucleotide bases long) situated within a longer RNA scaffold. The RNA scaffold binds to the targeted DNA, and the predesigned sequence guides the Cas9 enzyme to the right part of the genome and to cut at the correct targeted site. The gRNA consisting of complementary nucleotide bases is precisely designed to identify and bind to a specific targeted sequence in the DNA. The gRNA will theoretically bind to the target sequence only, but not to any other region in the genome.

Other Main Gene Editing Tools

Zinc finger proteins contain many finger-like structures linked via small peptide linkers in a tandem fashion. These proteins' tandem arrangement and their ability to recognize a wide variety of DNA sequences made them attractive to be bioengineered to design novel DNA-binding peptides/proteins. Zinc fingers are short stretches of amino acids folded into an ααβ compact structure with the help of a Zn ion. The Zn ion links the α-hairpin and β-helix in a tetrahedral fashion with the two conserved cysteine and two histidine residues. Although Zn metal is not directly involved in DNA-binding activity, it is critical for stability of the ααβ architecture of the zinc finger domain [14]. These bioengineered zinc finger DNA-binding proteins were used for targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations [15, 16]. Gene editing using ZFNs was many times more efficient in gene targeting and gene alteration via mutagenic DNA repair compared to conventional homologous recombination-based gene targeting. A milk protein, β-lactoglobulin (BLG) which is a primary milk allergen, is found in cow milk but not in human milk. Recently, BLG gene-free cows were generated using ZFN mRNA contributions [17]. In this study, primary female bovine fetal fibroblasts were transfected with designed BLG-ZFN mRNA, and BLG bi-allelic knockout cows were generated by somatic cell nuclear transfer (SCNT) [17].

Initial technologies such as ZFNs and TALENs suffer from lower specificity due to their off-target side effects. Many possible off-target genomic sites, based on high-sequence similarity to on-target sites, were identified in ZFN-mediated genome editing in human-induced pluripotent stem cells [18]. Another study targeting endogenous human chemokine (c-c motif) receptor 5 (CCR5) and vascular endothelial growth factor A (VEGFA) genes identified nine off-target sites by CCR5-induced changes and 31 off-target sites by VEGFA-induced alterations in cultured human cell lines [19]. Assembling zinc finger domains to bind a precise extended stretch of nucleotides with high affinity is not straightforward [20]. Bioengineering ZFNs to specific targets is difficult and time consuming. In addition, multiplex genome editing has been performed in only a few models using ZFNs and TALENs [21]. Further, the main obstacle was to develop a simple but effective delivery method for these proteins (ZFNs and TALENs) to enter the host cell compared to small gRNAs in the CRISPR-Cas9 system. Overall, CRISPR-Cas9 technology seems to provide better efficiency and feasibility, with greater suitability for multiple clinical applications.

Customized DNA-binding domains engineered using TALEN repeats were widely used in genome editing. TALENs consist of an engineered array of TALE repeats linked to the FokI nuclease domain. TALENs have been used to create site-specific DNA double-strand breaks in various organisms and cell types for directed genome editing [22]. Myostatin gene-edited cattle and sheep were produced by zygote injection of engineered TALEN mRNA [23] and they had advantages over the traditional process that involves generation of a knockout cell line using homologous recombination and SCNT into oocytes. Application of SCNT requires a high-level of technical expertise, reliable supply of oocytes, and many recipient animals. Myostatin knockout bovine fetal fibroblasts were obtained using TALENs and in which myostatin-TALEN vector was constructed and transfected into bovine fetal fibroblast cells.

Sequencing confirmed the knockout efficiency for TALEN-myostatin pairs and the efficiency was 20% [24]. In another elucidation, codelivery of myostatin and TALENs induced highly specific and efficient gene disruption in a variety of mouse, human, and bovine cells. This specific combination of myostatin and TALENs seemed to be very functional, based on sequence analysis and makes the application possible in many other mammalian species in the future [25]. One transgenic cow with site-specific marker-free *Sulfolobus solfataricus* β-glycosidase (LacS) was produced with a long-term purpose of generating cows producing low-lactose milk [26]. Site-specific integration of the LacS gene was mediated through TALEN-mediated microhomologous end-joining. The TALENs vector that targets the cow's β-casein locus, LacS gene expression vector that contains TALEN reorganization sequence, and microhomologous arms were constructed and cotransfected into bovine fetal derived skin fibroblasts. Positive cell clones were used as donors for SCNT and resultant embryos were transferred into recipient animals. LacS knockin rate in TALEN-treated cattle fetal fibroblasts was 0.8%. The blastocyst rate following SCNT was 27%, the three months pregnancy rate was 20%, and one transgenic female calf was born [26]. The prion protein (PRNP) gene that encodes prion protein was deleted by transfection of plasmids that encode TALEN pairs into bovine fibroblasts and the propagated cloned cells were used for SCNT [27]. Resultant blastocysts were confirmed to have deletion of the PRNP gene. TALEN-stimulated homology directed repair introduced a mini-gene donor construct, an 11.5-kb insert at the bovine albumin locus in primary bovine fibroblasts to create genetically modified cattle as a biopharming source of recombinant human serum albumin in the future and resulted in a targeting efficiency of 11%. Moreover, these manipulated fibroblast cells were used as nuclear donors in SCNT and developed to at least the blastocyst stage [28].

Although ZFNs and TALENs were used in the past for targeted genome editing, CRISPR has captured the attention of the research community, primarily due to its lower cost than TALENs and other genome-editing tools. Donor structure is simple and smaller in the CRISPR-Cas9 system (RNA–DNA) compared to donor structures in ZFNs and TALENs editing tools (protein-DNA) and this structure facilitates easier delivery of the CRISPR-Cas9 system into target cells. Versions of gRNA can be easily designed for any specific targets [29]. TALENs require complex cloning methods, whereas in CRISPR-Cas9 application, genes can be easily retargeted by standard cloning methods and oligo synthesis in most scenarios. Large-scale library preparation has not been performed using ZFNs and TALENs [30]. Although the techniques using ZFNs and TALENs have been improved by booming biotechnologies, and these methods are still being applied in certain situations, the CRISPR tool seems to be a game changer at present time pending discovery of another new technology, probably whole genome editing, to completely create synthetic animals and humans.

Reproduction and CRISPR-Cas9 Technology

Reproduction is the fundamental biological process by which organisms maintain their generations. Natural mutations in the DNA or RNA nucleotides, genes, or chromosomes can occur randomly, and these mutations may be beneficial or harmful with regards to inheritance and evolution. Gene editing is an intended genetic mutation in which DNA is inserted, deleted, modified, or replaced in the genome of a living organism. Whether it is a natural gene mutation or an intended change made in the gene, the reproductive process is critical to propagate the desired alteration in a population. DNA constructs harboring homologous stretches were able to enter, interact, and eventually integrate at the target site of the genome by the endogenous homologous recombination machinery in a variety of cells. Although gene editing by conventional homologous recombination has been successful in various somatic cells, DNA editing using conventional recombination techniques has been virtually unfeasible in germline, zygotes, embryo, and embryonic pluripotent cells. Nevertheless, discovery of novel and highly efficient DNA editing tools such as CRISPR-Cas9 technology has simplified genome editing in zygotes, germlines, and pluripotent stem cells.

Pronuclear and cytoplasmic microinjections of CRISPR ribonucleoprotein targeted tyrosinase gene by electroporation into murine zygotes were highly efficient, simple, and cost-effective, and facilitated indel mutations, exon deletions, point mutations, and small insertions [31]. To investigate the function of the pluripotent octamer-binding transcription factor 4 (OCT4) during human embryogenesis, the CRISPR-Cas9 system was used to manipulate OCT4 and this system efficiently and specifically targeted the gene encoding OCT4 in diploid human zygotes. Blastocyst development was compromised in OCT4-deleted zygotes [32]. Proto-oncogene, c-KIT, encodes the receptor tyrosine kinase and was better targeted using CRISPR-nuclease than utilizing CRISPR-mRNA in marmoset embryos, and mosaicism was avoided when the CRISPR-nuclease system was applied [33]. RNA-guided Cas9 nuclease genes efficiently facilitated genome editing in zebrafish embryos in a simple and robust manner when ETSRP, GATA4, or GATA5 genes were targeted. The Cas9-gRNA system induced target-specific indels in F0 mutants comparable to mutants produced with TALENs and ZFNs

in zebrafish embryos, making it feasible to generate heritable F1 mutants using the Cas9-gRNA system [34]. A protocol for microinjection of the CRISPR system into one blastomere of two-cell embryos was established to generate viable chimeric founder mice with a heritable embryonically lethal mutation to elucidate function of VIRMA and DPM1 genes [35]. Targeted deletion of the OCT4 gene was created by direct injection of the CRISPR/Cas9 reagent into bovine zygotes and disruption at the bovine OCT4 locus arrested embryonic development at the morula stage and hindered blastocyst formation [36]. When the FGF10 gene that encodes fibroblast growth factor 10 was targeted through microinjection of TALEN and CRISPR-Cas systems into one-cell mouse embryos, the resultant F0 phenotype from FGF10-deficient embryos had limb-defect. The CRISPR-Cas system produced limb defects with strikingly higher efficiency compared to the TALEN system [37].

Although scientists are successful in gene editing in mammalian zygotes and early embryos, the studies battle with manifold complexities of DNA repair in zygotes and early stages of embryonic development such as mosaicism and off-target effects. Many factors such as gRNAs, local chromatin effects, competition between Cas9 and endogenous transcriptional regulators, and epigenetic factors shape the success of gene modification in zygotes and early embryos. CRISPR-Cas9 is not immediately degraded after cutting and repairing the genome at the targeted site and may continue to slice off-target locations until its complete degradation. Synthesis of Cas9 protein from plasmids can lead to high enzyme concentrations, high levels of off-site targeting, and residual activity following desired gene manipulation. As an alternative, purified recombinant Cas9 protein can be directly injected into oocytes, zygotes, and embryos which may enhance specificity while shortening enzymatic exposure time, thus lessening off-site targeting. Also, limiting the CRISPR-Cas9 reagents or introducing an adjacent silent mutation effect in the donor DNA may prevent off-target and residual effects. Timing of editing in the embryo cannot be monitored and any editing that occurs after the first cell division is very likely to produce mosaicism in edited embryos.

Simultaneous injection of sperm and CRISPR reagents can reduce the level of mosaicism compared to the level that happens when the CRISPR reagent is injected after fertilization. The zygote will have more time to edit and complete repair before the first division when CRISPR component is micro-injected before fertilization. However, there will be residual activity later in the embryo if editing on one or both alleles is unsuccessful before the first cell division. Paternal and maternal chromosomes may be edited at various rates in the early embryo and repaired using different mechanisms. The paternal genome appears to be more efficient in gene editing than the maternal genome [32]. One allele sometimes remains to be unedited immediately after the first cell division. Alternative strategies might be to develop new protocols to edit germline cells before fertilization. In humans, gene repair in gametes or preimplantation embryos would reduce the risk of producing mutant embryos, thereby increasing numbers of embryos available for transfer and eliminating the need to dispose of a mutant embryo that is considered a potential life in some societies. Therefore germline gene therapy is more ethically acceptable to some families.

The CRISPR-Cas9 system was used to mutate an EGFP transgene and endogenous CRYGC gene in mouse spermatogonial stem cells (SSCs) without any off-targets. Mutated SSCs were transplanted into seminiferous tubules of infertile mouse testes to undergo spermatogenesis. Round spermatids were injected into mature oocytes to produce heterozygous offspring and these offspring displayed the corresponding mutant phenotypes [38]. The CRISPR-Cas9 provided a platform to generate targeted germline mutations in rat SSCs and to elucidate details of spermatogenesis in rats. In that study, EPSTI1 and ERBB3 genes were targeted to be modified, altered spermatogonial cells were transplanted into recipient rat testis to regenerate spermatogenesis, and pure non-mosaic mutant progenies were produced [39]. TALEN and CRISPR-Cas9 produced ROSA26 that is unrelated to spermatogenesis, and STRA8 that is indispensable to spermatogenesis, genes deleted mouse spermatogonial cells. Upon transplantation into a recipient testis, ROSA26 targeted cells produced competent sperm whereas STRA8 targeted cells showed defective spermatogenesis [40].

The CRISPR-Cas9 was used to manipulate the genome in zebrafish oocytes. Injecting oocytes in advance with predesigned CRISPR-Cas9 components and incubating them in oocyte incubation medium significantly improved efficiency of genome editing and germline transmission by *in vitro* fertilization in zebrafish compared to conventional methods. This novel strategy not only provided an efficient gene editing alternative but also generated heritable mutants in zebrafish [41]. The CRISPR-Cas9 targeted PDX1, a critical gene for pancreas development in sheep oocytes. Microinjecting CRISPR-Cas9 components in metaphase II oocytes was found to have reduced lysis, improved blastocyst rate, and increased the number of targeted biallelic mutations, and had similar degree of mosaicism compared to zygote microinjection of the same components. Also, a dual sgRNA system achieved complete PDX1 disruption and mutated fetuses with disrupted pancreatic development [42]. The CRISPR-Cas9 system can produce corrected mature sperm and oocytes that can be used to produce disease-free individuals. It has also been efficiently

applied to produce synthetic gene drives in populations to control genetic and vector-borne diseases. Safe applications of CRISPRs in reproduction can guarantee a huge reduction in genetic as well as vector-borne parasitic diseases in humans and animals.

The Cattle Industry and CRISPR-Cas9 Technology

In the cattle industry, selective breeding plays an important role in breeding superior males and females to produce progeny with specific characteristics or traits. The main goal of selective breeding is to reduce production costs and increase output values. Selective breeding started in the 1950s, to improve growth and conformation traits. Later, feed efficiency characteristics were paid attention to. Reproduction, meat quality, health, and behavioral traits were of interest in the 1990s in selective breeding programs. Current breeding programs also intend to select traits involved in longevity, metabolic disorders, infectious diseases, and climate tolerance. In the past, traditional selection was performed based on pedigree charts and phenotypes. A new development in genomics facilitated genotyping of animals. Single nucleotide polymorphisms (SNPs) and genome wide association studies helped to predict a phenotype associated with an SNP. Animals can be genotyped early in life, which can result in acceleration of genetic gain [43, 44].

In selective breeding, precise targeting of a trait is impossible. Selective breeding is a relatively slow and expensive process since it depends on natural selection and reproduction processes. Selective breeding can also result in harming animals. For example, selection to increase milk production in cattle resulted in discomfort for the animal because of the weight of huge udders and frequent milking. This caused inflammatory and metabolic diseases. Belgian Blue cattle are the result of selective breeding and in these cows the myostatin gene is mutated from the wild type, resulting in extreme muscle production [45, 46]. These cows have problems with calving and 80% of the calves are born via cesarean section. CRISPR-Cas9 is one of the modern gene editing tools; it simply speeds up selective breeding and the producer can choose exactly what genes need to be manipulated from a "catalog" of desirable traits, including reproductive traits in the cattle industry.

Japanese Black cattle, a beef breed in Japan, are known for the high quality and distinctive marbling characteristics of their meat. Several years of selective breeding not only generated this beef breed with high quality and very palatable meat, but also resulted in accumulation of recessive mutations that cause genetic diseases. CRISPR-Cas9-assisted genome editing corrected isoleucyl-tRNA synthetase (IARS) syndrome which is a recessive disease in Japanese Black cattle caused by a single nucleotide substitution [47]. In this study, CRISPR/Cas9, donor DNA containing a codon for the correct amino acid, and a green fluorescent protein were introduced into bovine fetal fibroblast cells isolated from a homozygous mutant calf. Enriched recombinant cells with the repaired allele were cloned to produce SCNT embryos. Twenty-two cloned blastocysts were transferred to recipient cows, with the success of five viable fetuses at days 34 and 36. Further fetal genomic DNA analysis exhibited the correct repair of the IARS mutation without any other abnormal DNA footprint [47].

Disbudding of calves (destruction of the cells that produce horns before they grow and attach to the skull) is a routine practice in the US dairy cattle industry to prevent injury to other animals and handlers, reduce feeding trough space, and diminish aggressive behaviors. However, the procedure of disbudding/dehorning is a painful process for the cattle and an unpleasant task for the personnel. Therefore alternative, more humane solutions including precision gene editing and breeding have been sought to flawlessly integrate the polled trait into high genetic merit dairy cattle. In this instance, the horned gene is replaced with a naturally occurring polled gene to produce naturally hornless calves, considering animal well-being and health.

A bovine POLLED allele was introgressed into fibroblasts obtained from horned bulls using TALENs to introduce a hornless phenotype into dairy bulls, and two hornless dairy bulls were subsequently produced by cloning [48, 49]. Following this gene editing task, a recent study [50] investigated genomic and phenotypic analyses of offspring produced by crossing one of the hornless bulls with horned cows to determine whether the polled genome edit was passed on to offspring. The genome-edited dairy bull had the homozygous dominant POLLED allele whereas horned cows had the homozygous recessive allele. Six heterozygous polled calves were born and all were healthy and phenotypically unremarkable. These calves are apparently the first reported offspring of a genome-edited bull. However, based on whole genome sequencing of all animals using an Illumina HiSeq4000, it has been revealed that two calves inherited the naturally occurring hornless allele and four calves additionally inherited a fragment of bacterial DNA, known as a plasmid, used to stimulate homologous directed repair while delivering the hornless trait to the bull. This fragment of bacterial DNA had integrated alongside one of the two hornless alleles in the bull. Although plasmid integration can be screened and the genome-edited hornless bull that inherited only the naturally occurring allele without any remnant of plasmid

DNA can be selected, the US Food and Drug Administration (FDA) may consider these bulls and offspring as genetically modified organisms since they contain foreign DNA, bacterial plasmid DNA. New alternative methods that no longer use foreign DNA sequence in the donor template to deliver the hornless allele should be sought to fulfill the regulatory guidelines.

The CRISPR-Cas9 system was used to elucidate the function of POU domain, class 5, transcription factor 1 (POU5F1) in bovine embryos [36]. POU5F1 encodes a transcription factor containing a POU homeodomain that has critical roles in maintaining stem cell pluripotency and embryonic development. In this study [36], a synthetic gRNA targeting bovine POU5F1 exon 2 along with Cas9 protein were microinjected into the cytoplasm of bovine zygotes. Targeted mutation of embryonic POU5F1 exon 2 was highly efficient, with large deletions, resulting in a complete knockout rate of 86 and 78% bi-allelic deletions. Upon further observations of CRISPR-induced POU5F1-altered and control bovine embryos, it was concluded that POU5F1 expression is required for blastocyst and expanded blastocyst formation but not for development to the morula stage [36]. Single Cas9 nickase (Cas9n) created a single strand break and inserted a gene, the natural tuberculosis resistance-associated macrophage protein-1 gene, at a selected locus in bovine fetal fibroblast cells, albeit with some off-target effects. Subsequently, embryo transfer followed by SCNT generated transgenic cattle with increased resistance to tuberculosis [51].

Fertility is a key component of modern cattle production systems. Reproductive traits are multifactorial in nature and largely influenced by multiple genes and environment. In the past, intensive selective breeding for production traits negatively influenced fertility traits such as ovulation, estrus expression, fertilization, and embryonic survival. With the capability of CRISPR-Cas9-mediated multiplex gene editing, multiple genes and several traits can be precisely and simultaneously targeted, avoiding these negative additive effects.

Limitations of CRISPR-Cas9 Applications

Although the CRISPR-Cas9 system is currently the method of choice for genome editing in animals, it also comes with some technical limitations. Several parameters that control efficiency of CRISPR-Cas9-mediated targeted deletion, insertion, and alteration are yet to be completely understood. Unfortunately, a challenging technical limitation – presence of more than one genotype which is called genetic mosaicism – in an individual following microinjection of CRISPR into zygotes is commonly seen [52–55]. This limitation is sometimes overlooked and occasionally impeded targeted mutagenesis by its direct application into embryos. Early microinjection (10 hours post insemination [hpi]) of the CRISPR-Cas9 system into zygotes and microinjection of the CRISPR-Cas9 system into oocytes before fertilization (0 hpi) significantly reduced the rate of mosaicism compared to the standard 20 hpi protocol group [56]. Promoting Cas9 degradation by combining Cas9 with ubiquitin reduced genetic mosaicism in non-human primate embryos and enhanced precision genome editing [57]. Wild-type Cas9 compared to other variants, and pronuclear injection of the CRISPR-Cas9 system compared to cytoplasmic injection reduced the rate of mosaicism in mice zygotes [58].

There are numerous disease-causing single-gene mutations in humans and animals and these gene mutations can be often corrected using CRISPR-Cas9 applications in human zygotes. The Cas9-sgRNA complex creates a DNA double-strand break at the targeted site that stimulates the endogenous DNA repair system, resulting in targeted genome modifications. Even though CRISPR-Cas9 offers a tangible potential to correct genetic defects in humans and animals, effectiveness of correcting genetic defects via CRISPR technology in mammalian zygotes has not been completely elucidated apart from its merit and ethics. Homologous directed repair efficiency is found to be variable. The CRISPR-Cas9 system had the ability to cleave the endogenous β-globin gene in tripronuclear human zygotes, although homologous recombination directed repair lacked efficiency. Several edited embryos also had mosaicism, and off-target cleavage was apparent in some of these zygotes [59]. Moreover, the endogenous Δ-globin gene which is like the β-globin gene competed with exogenous donor oligos to function as the repair template, resulting in untoward mutations. Naturally occurring CCR5Δ32 allele was successfully replaced in early human 3PN embryos by co-injecting Cas9 mRNA, gRNAs, and donor DNA. However, in the edited embryos containing the engineered CCR5Δ32 allele, function of the other alleles at the same locus could not be completely regulated because of untoward indel mutations [60]. To address off-target effects, untoward mutations, and mosaicisms the CRISPR-Cas9 system and protocols of gene editing should be continuously improved. Single-point mutations, in which one single nucleotide base is added, deleted, or altered in the DNA, are commonly seen in genetic diseases affecting humans and animals. A pristine gene editing technique known as prime editing can rewrite the DNA by only severing a single strand to insert, delete, or replace a base, and is being tested in human and mouse cells [61]. This prime editing tool has a catalytically modified Cas9 fused to an engineered reverse transcriptase and programmed with a prime

editing guide RNA (pegRNA). This tool can specifically target the single point mutated site and encode the desired edit better than the existing genome-editing approach of CRISPR-Cas9 that performs a double strand cleavage.

Despite these limitations, CRISR-Ca9 has several advantages. Gene editing is more efficient using CRISPR-Cas9 compared to other major applications. The protocol is very simple and relatively inexpensive. Furthermore, sensitivity is high as CRISPR-Cas9 has sequence-specific gene targeting. Multiplexed genome editing is possible with application of CRISPR-Cas9 as multiple sgRNAs of several genes can be targeted simultaneously [62]. With the use of the CRISPR-Cas9 tool, the disadvantages of current selective breeding programs can be addressed. As well, CRISPR-Cas9 technology could be used in combination to complement current breeding programs to address these negative factors and to increase reproduction and production efficiencies and farm profits. Regardless, a stringent approval process for gene-edited animals and animal products, and public perception may restrict the use of targeted gene editing.

US Regulation of Gene-Edited Food Animals

Millions of naturally occurring DNA variations are seen in a population of a given animal species. These variations occur at a slower rate and allow breeders to select a desirable variation for selective breeding. Sometimes these naturally occurring variations happen incorrectly and result in an undesirable trait. However, nobody controls naturally occurring mutations in plants and animals and no regulatory agencies appraise them before they come to market. In contrast, discovery of new gene editing tools now allows us to precisely introduce the useful genetic variation into agricultural breeding programs and propagate the desirable trait significantly faster. Nevertheless, regulations come into effect when gene editing technologies are used to generate a genetic variation regardless of its benefit. The US Department of Agriculture (USDA) considers gene-edited plants and crops as being like plants and crops produced through conventional breeding techniques. Therefore gene-edited plants do not require additional regulatory oversight prior to commercialization as opposed to genetically modified organisms that contain exogenous genes from other species such as bacteria. With this regulatory protocol, USDA supports innovation when there is no risk present in the products. However, due to the complicated framework in the USA, the FDA plays a role in regulating animals and animal products for human consumption, has stringent guidelines in evaluating gene-edited animals and animal products, and undertakes mandatory premarket evaluation for all food animals whose genomes have been intentionally altered using modern molecular technologies, including gene editing technologies [63, 64]. The FDA views these products as new animal drugs requiring regulatory oversight. This rigorous regulation goes against the already developed US biotechnology policy that regulatory oversight should be triggered by unreasonable risk in the product and not by the fact that an organism has been modified by a process or technique. Regulations on gene-edited food animals and animal products by the FDA do not fulfill the purpose of biotechnology and advancement. Therefore harmonizing the regulations associated with gene editing in food species is critical to allow both plant and animal breeders access to advanced gene editing tools to introduce beneficial sustainability traits like disease resistance, climate tolerance, and attributes to improve food quantity and quality within US agricultural breeding programs.

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Bovine Reproduction, Second Edition. Edited by Richard M. Hopper.
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