1. Introduction

The stallion represents one half of the breeding equation; however, apportioned time and funding has been considerably less for efforts to advance the discipline of stallion reproduction compared with that of mare reproduction. Modern-day fathers of stallion reproduction, such as Robert M. Kenney, Bill W. Pickett, and Marion Tischner, made significant strides in our understanding of stallion reproductive physiology and clinical disease, but this fascinating area of study begs for individuals with the aptitude and tenacity required to take the discipline to new heights. In recent years, the level of interest in research directed toward the breeding stallion has increased noticeably, as evidenced by the 2006 Congress of the International Society of Equine Reproduction (ISER). The stallion was represented in the largest section of papers presented and published at this meeting, a first since the inception of the ISER in 1975.

This paper will be directed primarily at the male gamete, the spermatozoon, and all the fascination that it bestows on those of us that have dedicated a large portion of our professional lives eavesdropping on its microcosm. We will begin with a detailed overview of spermatozoal structure and function and the events that accompany a spermatozoon’s sojourn through the female reproductive tract, followed by a discussion of some potential clinical ramifications. The lecture that accompanies this text will be directed, almost exclusively, to practical applications and advancements in the discipline of stallion reproduction. Not all published work could be represented in this short review, and we preface this reading with an apology for unintentional exclusion of pertinent data. Although we direct the reader to some relevant studies conducted in equids, most of the information provided in this manuscript stems from studies conducted in other species, where the process of discovery has been most pronounced. Although this comparative approach can be quite enlightening, a drawback is that the relevance to stallions of findings unveiled in other species will require documentation.

A manuscript directed exclusively at the spermatozoon would seem to represent a miniscule part of the total reproduction picture. On close scrutiny, however, one becomes enlightened about the level of scientific interest in this cell. As an example, ~50,000 entries are logged in response to a query for the keywords, spermatozoa or spermatozoon, in PubMed, a database of indexed citations offered by
the National Institutes of Health. An uninformed individual might describe a spermatozoon as a highly specialized, but simple, cell with only one role to fulfill—that of fertilization. Although fertilization is the endpoint of spermatozoal function, this cell must be extremely sophisticated and adaptable to achieve this task, and the process involves a series of highly coordinated cellular and molecular events. The following is a list of some requirements ascribed to a mammalian spermatozoon.

1. Loss of most organelles and cytoplasm during formation in the testis and maturation in the epididymis (requires a host of intracellular and intercellular signaling events)

2. Remodeling of spermatozoal chromatin within the epididymis as a protective mechanism against environmental injury (requires repackaging of nuclear DNA into a highly condensed form through the aid of specialized proteins termed protamines)

3. Plasma membrane alterations within the epididymis to yield proteins important to fertilization (requires various enzymatic-linked alterations of existing proteins and uptake of proteins from epididymal fluid or from the epididymal epithelium)

4. Passage through the uterus and uterotubal junction of the female at the time of insemination (requires activated flagellar movements and protection against immunologic attack)

5. Binding to oviductal epithelial cells to form a spermatozoal reservoir (requires specific cell-cell attachment, possibly mediated through spermatozoal surface carbohydrate-binding proteins, termed lectins)

6. Acquisition of additional maturation changes, collectively termed capacitation, that permit a spermatozoon to fertilize an oocyte (requires an assortment of signal transduction cascades)

7. Release from oviductal epithelial cells and passage to the vicinity of the oocyte at the isthmic-ampullar junction of the oviduct (requires a coordinated spermatozoal-release mechanism, hyperactivated motility, and probably chemotaxis)

8. Penetration through the extracellular matrix of the oocyte cumulus (possibly mediated by hyperactivated motility and redistribution/unmasking of surface-associated hyaluronidase, because the cumulus matrix is rich in hyaluronic acid)

9. Binding to the zona pellucida, a highly glycosylated protein matrix surrounding the oocyte (seems to involve specific affinity between spermatozoal surface molecules and the zona pellucida components)

10. Acquisition of the acrosome reaction, a regulated form of exocytosis (requires reorganization of the outer acrosomal and overlying plasma membranes necessary for fusion and vesiculation)

11. Penetration of the zona pellucida (release of acrosomal proteins with enzymatic activity is required for this event to occur)

12. Binding and fusion with the oolemma (requires specific region-dependent molecular interactions)

13. Dispersion of nuclear contents (requires specific fusogenic alterations of the lipid membranes of the spermatozoon and oocyte)

14. Oocyte activation (a spermatozoon-derived factor is required for activation of the oocyte and embryonic development)

15. Pronucleus formation (requires decondensation of the highly compact spermatozoal nucleus)

16. Organization of the mitotic spindle after pronuclear formation (requires contribution of proximal centriole from the spermatozoon)

After viewing this lengthy list of functions, one becomes quite appreciative of the highly complex and specialized features of a spermatozoon. In fact, the biochemical and biophysical features are so sophisticated that many of the cellular and molecular mechanisms remain unresolved to this day. Furthermore, spermatozoa (and oocytes) represent some of the most highly differentiated cells in the mammalian body; yet, when sperm and oocyte are combined, they retain their potential for totipotency (ability to divide and produce all cell types of the body) through creation of a zygote.

2. Origin of the Spermatozoon

The life of a spermatozoon begins within the testes, unless, of course, one wishes to consider the embryonic origin of the primordial germ cells. The testes, an elaborately designed organ, is classically considered to possess two functions: (1) exocrine—spermatogenesis, and (2) endocrine—production of hormones important to spermatogenesis, sexual differentiation, development of secondary sex characteristics, and libido. Although this simplistic description provides one with the general concept of testicular function, it does not portray the extremely complex nature and elegant interplay of these two processes. Conventional descriptions convey the role of hypothalamic- and pituitary-derived hormones on regulation of testicular function, as well as feedback mechanisms required for homeostasis. Although such pathways are undoubtedly the key orchestrators of testicular function, emerging information is revealing a multitude of subcellular, molecular-mediated events that “cloud” our understanding of the events that actually occur within the testes. As with any area of study, the more learned we become about a topic, the more queries surface that require additional clarification. Such is the case with testicular function. Without
question, a thorough understanding of testicular function will require a keen appreciation of the mechanisms by which genes and gene products are expressed and repressed. As an example of the genetic complexity surrounding control of testicular function, new information has revealed that single nucleotide polymorphisms (SNPs) have been identified in the follicle-stimulating hormone (FSH) receptor gene of humans, resulting in a mutation of the FSH receptor (as is found on the surface of the Sertoli cell) that can influence its activity. As another example, use of transgenic mice deficient in estrogen receptor genes has shown that estrogens are likely to play a more important role in testicular function than was once thought to be the case. Similarly, use of mice with a selective androgen receptor knockout in Sertoli cells revealed that the androgen receptor in the Sertoli cell is an absolute requirement for normal spermatogenesis. Furthermore, experimentation with germ cell–specific androgen receptor knockout mice revealed normal spermatogenesis, suggesting that germ cell androgen receptors may play different roles as the germ cells progress through spermatogenesis. As seen here, to more fully understand what makes the testes tick, we must capitalize on the powerful molecular tools that have been developed in this capacity. Most of these types of studies are conducted in human and laboratory specimens, so it will be important to test the relevance to the horse.

Organization of the Testis
The testes are composed of testicular parenchyma encapsulated by the thick fibrous tunica albuginea. Extensions of the tunica albuginea penetrate the underlying testicular parenchyma, dividing it into numerous lobules (Figs. 1 and 2). The tunica albuginea is composed of collagen and elastic fibers, myoid cells, and a network of blood vessels (Fig. 3). Testicular parenchyma occupies nearly 90% of the total testicular mass in adult horses, and >70% of the testicular parenchyma is occupied by seminiferous tubules. The interstitial component of the testis is located between seminiferous tubules and consists primarily of the Leydig cells intermingled with fibroblasts, lymphocytes, mast cells, blood vessels, lymphatic vessels, and extracellular matrix (Figs. 4–10). Nerves are rarely seen in the testicular interstitium, but they are well established in the area of the spermatic cord leading to the testis.

Spermatogenesis
Spermatozoal production, i.e., spermatogenesis, occurs within the seminiferous tubules. Both ends of these highly coiled tubules open directly into the rete testis, such that the products (both cellular and non-cellular) of the seminiferous tubules are excreted into the rete testis and delivered to the efferent duct system (e.g., the epididymis and ductus deferens). The numerous and tortuous seminiferous tubules, with a combined average length of 2419 m per testis in the stallion, are comprised of an epithelial wall, termed the seminiferous or germinal epithelium, and a lumen. The seminiferous epithelium consists of germ cells in various steps of development intermingled with Sertoli cells that serve to provide structural support and a nurturing source to the germ cells. The Sertoli cells are anchored to the basement membrane, and extend to the lumen, of the seminiferous tubules. The semi-
niferous tubules are bordered by peritubular myoid cells (myofibroblasts) that, through peristaltic contractions, may aid in evacuation of luminal contents into the rete testis. These myoid cells are also considered to be involved in paracrine signaling events.21–23

A critical feature of the seminiferous epithelium is the formation of tight junctional complexes that develop between Sertoli cells, thereby physically dividing the seminiferous epithelium into basal and adluminal compartments (Fig. 11).17 This structural complex is termed the blood–testis barrier because it restricts direct access of blood-borne substances into the adluminal compartment. The described actions of the blood–testis barrier are (1) to segregate meiotic (except the earliest preleptotene spermatocytes) and post-meiotic germ cells from immunologic attack, because these germ cells are considered to be in an immunologically privileged site, and (2) to provide a unique microenvironment for the final stages of germ cell development within the adluminal compartment.24 Although several blood barriers exist in the general body, there is no counterpart to the blood–testis barrier in the female. In the female, development of primary oocytes occurs before the immune system recognizes self, and the female does develop the counterpart to spermatids (i.e., ootids). In the male, germ cell development to spermatocytes, and spermatids does not occur until puberty. This is well after the immune system recognizes self and considers these cells as foreign. Especially intriguing is the molecular control over the transient disassembly of the blood–testis barrier to facilitate migration of germ cells from the basal into the adluminal compartment.24,25

Spermatogenesis is an extremely complex process that involves germ cell proliferation, germ cell differentiation, and, paradoxically, programmed germ cell death (termed apoptosis). This lengthy process, 57 days in the stallion,20,26 is controlled by a vast array of messengers acting through endocrine, paracrine, and autocrine pathways.27–31

Spermatogenesis not only involves transformation of undifferentiated diploid germ cells into highly differentiated and specialized haploid spermatozoa (Figs. 12 and 13),17,26 but it also involves profound transcriptional modifications within the cells.3,32
As a partial list, the finished product of spermatogenesis has (1) a $1/8$ chromosomal complement that is profoundly repackaged, (2) a newly formed and intricately designed flagellum, (3) the biogenesis of a highly complex secretory vesicle, the acrosome, and (4) retention of some mRNA (in a largely depleted cytoplasmic package) that likely has implications in fertilization and post-fertilization events.

Spermatogenesis is initiated by the differentiation of spermatogonia from a stem cell pool that is continually replenished for most of a stallion’s adult life. Under normal circumstances, this is a highly productive process, yielding on the order of $5–6$ billion spermatozoa per day for an adult stallion. This translates into $30–40$ trillion spermatozoa produced over the course of a stallion’s life. From an...
equally impressive view, an average healthy stallion produces 60,000–70,000 spermatozoa per second. These newly formed spermatogonia enter a proliferative phase, whereby continuous mitotic amplifications yield a dramatic increase in spermatogonial numbers. Interestingly, the cytoplasmic component of mitotic divisions is often incomplete, resulting in daughter cells that remain connected by intracytoplasmic bridges (Fig. 14). Such an arrangement permits direct communication within this syncytium of developing germ cells, thereby assisting in their synchronous development. After completion of spermatocytogenesis, the spermatozoa enter a meiotic phase, characterized by duplication and exchange of genetic information (i.e., genetic recombination) and two meiotic divisions that reduce the chromosome complement to form haploid round spermatids. It is during the meiotic stage that germ cells pass through the blood–testis barrier to enter the adluminal compartment. During the final phase of development, spermiogenesis, the round spermatids undergo a dramatic transformation that includes nuclear reshaping through chromatin compaction, creation of a flagellum, development of the acrosome, and considerable loss of cytoplasm (Figs. 13 and 14). The fully developed spermatids are released as spermatozoa into the lumen of the seminiferous tubules by a process termed spermiation (Fig. 15). It is during spermiogenesis that the germ cells may be most vulnerable to both structural and genetic defects.

Cellular Associations During Spermatogenesis

The spermatogenic cycle, or cycle of the seminiferous epithelium, represents the series of changes in a given region of a seminiferous tubule between two appearances of the same developmental stages. For instance, if spermiation were used as a reference point, the cycle would consist of all the cellular associations occurring within a given cross-section of a seminiferous tubule between two consecutive spermiations (Fig. 16). These cross-sectional cellular associations can be divided into eight distinct stages (Figs. 16–19). The spermatogenic cycle length is constant at 12.2 days in the stallion. The spermatogenic wave, on the other hand, refers to the spatial, sequential order of stages along the length of a seminiferous tubule at a given point in time (Fig. 20). These two characteristics of the seminiferous epithelium can be defined because of the synchronous nature of development for cohorts of differentiating germ cells along the length of the seminiferous tubules. Histologic evaluation of the stages of the seminiferous epithelial cycle permits one to assess the efficiency of spermatogenesis.

Germ Cell Degeneration

Germ cell degeneration can be amplified in stallions with abnormal testicular function, but it is also a normal phenomenon in spermatogenesis. In fact, “normal” spermatogenesis is a relatively inefficient process and has been reported to result in an estimated loss of 25–75% of the potential number of spermatozoa produced by spermiation in the rat. In the stallion, germ cell degeneration is more profound during the physiologic breeding season than during the non-breeding season. Developing spermatogonia are the most vulnerable to apoptotic degeneration (Fig. 21). It is possible that the “physiologic” form of germ cell degeneration is a homeostatic mechanism that prevents overloading the Sertoli cells, i.e., to maintain a fine balance in the germ cell:Sertoli cell ratio. Apoptosis is a well-defined physiological process of cell elimination, and the apoptotic process is required for normal spermatogenesis in mammals. A protective role of luteinizing hormone (LH) against germ cell apoptosis has been reported in rats, based on expression of various apoptotic genes following immunoneutralization of LH in germ cells. Apoptosis of spermatogonia and spermatocytes has been reported in normal stallion testes, a finding that is consistent with the expected time that germ...
cells may be susceptible to removal from the system. Of interest, formation of the developing seminiferous tubules and the Sertoli cell (i.e., blood–testis) barrier coincides with increased germ cell apoptotic rates in stallions, providing evidence that apoptosis may play an intricate role in initiation of spermatogenesis. As expected, these changes are coincident with gene expression patterns. Studies involving rats indicate that FSH is an important regulator of this event.

3. Epididymal Transit and Maturation
Mammalian spermatozoa are incapable of in vivo fertilization on exiting the testis. The cells must undergo considerable post-testicular remodeling within the epididymis to acquire this ability. Each epididymis is an unbranched tortuous duct that spans 70–80 m in the stallion. The epididymis is connected to the rete testis by several efferent ducts (Fig. 2). The anatomical features of the rete testis and efferent ductules have been well characterized in the stallion (Figs. 22–24). For descriptive purposes, each epididymis is typically divided anatomically into a caput (or head), a corpus (or body), and a cauda (or tail; Fig. 2). The caput is closely applied to the cranial pole of the testis, the corpus courses over the dorsolateral surface of the testis, and the bulbous cauda is attached loosely to the caudal pole of the testis through the proper ligament of the testis (Fig. 1). An initial segment and an intermediate zone have also been ascribed to the beginning portion of the epididymal duct within the caput.

On average, 90 billion spermatozoa reside in the excurrent ducts of reproductively mature stallions when sexually rested, with a lower number (60 billion) reported for 2- to 4-yr-old stallions. On average, 62% of the spermatozoa within the excurrent ducts reside in the rete testis, with the remainder present in the epididymis. The epididymis proper contains 26% of the spermatozoa, the caput 4%, and the corpus and cauda 3% of the spermatozoa. The volume of the epididymal ducts within the rete testis is 1.4 mL, with the caput containing 1.0 mL, the corpus 0.3 mL, and the cauda 0.1 mL of spermatozoa. The epididymis is a specialized storage area for spermatozoa, where they undergo maturation and acquire their ability to fertilize oocytes.
duct system are in the caudae epididymides, and 7% are in the ductus deferens.\textsuperscript{59} Based on radiolabeled studies, the average time required for spermatozoal transit through the epididymis of the stallion is reported to be 8–11 days.\textsuperscript{60} Spermatozoal transit time through the caput and corpus segments is constant at 4 days and is unaffected by frequency of ejaculation, age, or season,\textsuperscript{60–62} whereas transit time through the cauda epididymis can be accelerated by increased ejaculation frequency.\textsuperscript{61,63} Spermatozoal number in the caudae epididymides and ductus deferens (i.e., spermatozoa available for ejaculation) may be reduced by \textasciitilde30% when semen is collected once every 2 days compared to sexually rested stallions.\textsuperscript{59} The normal spermatozoal transit time through the cauda epididymis of the stallion ranges from 4 to 7 days. Spermatozoa enter the epididymis at a constant rate in reproductively normal stallions (average of \textasciitilde5 billion spermatozoa per day). Spermatozoal phagocytosis within the epididymis is considered to be rare,\textsuperscript{58,63} so spermatozoa must also exit the epididymis at a similar rate to prevent epididymal overload. The length of time that spermatozoa can remain in the cauda epididymis without suffering storage-related injury has not been critically studied. Under normal conditions in sexually rested stallions, rhythmic contractions of the smooth musculature in the caudae epididymides and deferent ducts are thought to result in spontaneous emission of spermatozoa into the urethra. Some stallions are known to accumulate excessive numbers of spermatozoa in the caudae epididymides, deferent ducts, and ampullar luminae (the ampullae surround the terminal segment of the ductus deferens). This condition, termed “spermiosis”\textsuperscript{64} or “plugged ampullae”\textsuperscript{65} is thought to be associated with an intrinsic dysfunction in contractility of the smooth musculature surrounding the cauda epididymis and ductus deferens. It leads to a marked reduction in spermatozoal quality or azoospermia if the ductal luminae become obstructed completely.\textsuperscript{14} A separate subset of stallions is known to experience reduced semen quality and fertility if sexually rested for \textasciitilde12–24 h (unpublished observations). In this instance, the microenvironment within the cauda epididymis may impart factors that impede spermatozoal survival rather than favoring maximal survival time. Because epididymal spermatozoa are known to be susceptible to oxidative injury,\textsuperscript{66,67} it is possible that reduced elimination of potentially harmful agents or reduced synthesis and secretion of antioxidants contributes to this disorder.\textsuperscript{58} Alternatively, spermatozoal membranes that are overly abundant in polyunsaturated fatty acids may be at increased risk of oxidative injury.\textsuperscript{67}

As is the case with many other species studied, stallion spermatozoa are immotile upon entering the...
epididymis and do not gain motility until reaching the distal corpus. A motility pattern consistent with ejaculated spermatozoa is not acquired until spermatozoa reach the cauda epididymidis. As such, movement of spermatozoa through the epididymis is primarily attributed to rhythmic contractions of the smooth musculature that surrounds the epididymal duct (Figs. 25–27). In men, both the thickness of the smooth musculature, and its adrenergic innervation, increase from the proximal to the distal end of the epididymis, and into the ductus deferens. Autonomic drugs, both cholinergic and adrenergic, increase contractility of all segments of the epididymis. Prostaglandins (PGF2α and PGE) have been isolated from the testes and excurrent ducts of male rats, with a concentration noted to be lowest in the testes, intermediate in the epididymides, and highest in the ductus deferens. These same authors showed that aspirin, which suppresses production of PGF2α, strongly inhibited contractility of the caput epididymidis in vitro. Oxytocin receptors are also present in the epididymal epithelium, and the frequency of caput epididymal contractions increases and tubule diameter decreases in a dose-responsive manner to oxytocin exposure. Although the epididymis is considered to be an androgen-dependent tissue, estrogens seem to play a more important role than androgens in promotion of epididymal motility. This is achieved through estrogen-mediated up-regulation of both the oxytocin receptor gene and the receptor protein.

In addition to serving as a conduit, the epididymis bestows on spermatozoa specific structural and physiologic alterations, the result of which yields acquisition of fertilizing potential. Maturational changes in spermatozoa occur predominantly within the caput and corpus, because spermatozoa recovered from the cauda have attained a fertilizing capacity similar to that of ejaculated spermatozoa for most species studied. The cauda epididymis is considered to be a storage reservoir for spermatozoa, but this capacity extends into the ductus deferens and ampullae. In one study, sufficient spermatozoa were present in the ductus deferens and ampullae of stallions to account for average spermatozoal numbers in an ejaculate. A paucity of information exists with regard to the specific mechanisms that control spermatozoal maturation. Studies to date indicate that a salient fea-

Fig. 11. Transmission electron micrograph of Sertoli cells and germ cells and Sertoli cell–Sertoli cell junctional complexes (inset). Junctional complexes (JCs) between adjacent Sertoli cells (SCs) separate the basal compartment that contains spermatogonia (Sg) from the adluminal compartment that contains primary spermatocytes (PSs) and spermatids (St). Junctional complexes between adjacent Sertoli cells in the stallion are similar to those of other mammals. These include fusion (F) of outer leaflets of the plasma membranes of two Sertoli cells and the presence of juxtaposed endoplasmic reticulum (ER) and bundles of fine filaments (BFs). Scale bars = 1 and 2 μm (inset).
tecture of the epididymis is a continual change in the microenvironment to which spermatozoa are exposed during epididymal transit. This is portrayed by the variation in histomorphologic characteristics of the numerous epididymal cell types that line the various regions of the duct (Figs. 24–27), as well as the distinct regional and cell-specific patterns of gene expression and product secretion.

Although a variety of such localization studies have been conducted, these must be followed by mechanistic and functional approaches before we can fully appreciate the molecular interactions that impart fertilizing power to a spermatozoon.

The unique composition of epididymal plasma can be attributed, in large part, to the formation of a blood–epididymis barrier. This barrier is formed by tight junctional complexes near the luminal border between adjacent principal cells of the epididymal epithelium. This mechanism restricts entry of various blood-borne molecules into the epididymal lumen, thereby allowing spermatozoa to be bathed in a milieu that is controlled locally, and affording spermatozoal protection against immunologic attack. Disruption of the blood–testis barrier and the blood–epididymis barrier may be a contributor to reduced semen quality in older males. The corpus may be the most vulnerable segment of the epididymis to age-related barrier dysfunction.

Barrier-mediated isolation of the epididymal lumen from the blood plasma probably necessitates more reliance on alternative signaling pathways. Paracrine pathways (where target cells in the epididymis are issued signals from secretions of neighboring cells) and juxtacrine pathways (where target cell membrane receptors are activated by molecules on the plasma membrane of neighboring cells) are likely to exist. Lumicrine pathways (where signaling molecules originate in the testes and are transported through the rete testis into the epididymal duct or originate in one region of the epididymis and have an effect in another region of the excurrent duct system) are also key to epididymal function. Testosterone (synthesized by the Leydig cells) and androgen binding protein (synthesized by the Sertoli cells) represent prime examples of a lumicrine-derived mechanism of action in the epididymis. Of interest, androgen binding protein can also be synthesized and secreted by principal cells along the entire length of the epididymis, possibly exerting a paracrine- or lumicrine-related action.

An assortment of growth factors derived...
from the testis have been proposed to have an action on the epididymis in a lumicrine fashion, and many growth factors are also produced locally within the epididymis. Close interactions between spermatozoa and the epididymal epithelium are crucial for the maturation process to occur, so it is not surprising that spermatozoa may also serve a lumicrine role within the epididymis.

In summary, the epididymis serves to concentrate, mature, transport, and store spermatozoa. Massive resorption of luminal water within the efferent ductules and proximal caput and, to a much lesser extent, in the remainder of the epididymal duct leads to a $10^5$ increase in spermatozoal concentration in the cauda epididymidis compared with the rete testis. Observable changes in spermatozoa during epididymal maturation, based on light microscopic analysis, include (1) acquisition of flagellar movement within the corpus epididymis, followed by a progressive pattern of spermatozoal motility within the corpus epididymis, and (2) translocation and shedding of the cytoplasmic droplet. The cyto-

Fig. 13. Transmission electron micrographs of equine spermatids representing different steps of development during spermiogenesis. (A–E) The Sa spermatids (Sa) are characterized by a large Golgi apparatus (GA) that produced vesicles (Vs) that fuse to form larger vesicles and ultimately to form the acrosomic vesicle (AV). The acrosomic vesicle indents the nuclear envelope and flattens over the nucleus. (B) Developing flagella (F) from neighboring Sa spermatids have extended away from the spermatid cell body into the extracellular space between the Sertoli cell (SC) and spermatid. (C) Cytoplasmic bridges connect adjacent spermatids. (F) In the Sb1 spermatids (Sb1), the acrosome had formed a head cap over the nucleus. (G and H) The Sc spermatid (Sc) has a distinct manchette (Mn), elongating and condensing nucleus, attached flagellum with distinct annulus (An), and well-defined acrosome (A) over the anterior portion of the nucleus. (I and J) The Sd spermatids (Sd1 and Sd2) have lost their manchette and have further condensation of the nucleus. (B) The formation of the flagellum begins in the early Sa spermatid (G and H) appears as a growing axoneme in Sc spermatids, but (I) develops outer dense fibers (DFs), the fibrous sheath (FS), and has mitochondrial migration around the flagellum in early Sd1 spermatids. (J) Late Sd2 spermatids are mostly extended into the lumen (TL) and have a completely formed flagellum, with mitochondria (M) surrounding the middle piece and a distinct fibrous sheath (FS). Excess cytoplasm of the spermatids remains in the proximal region of the middle piece as a cytoplasmic droplet (CD). Scale bar = 2 μm.17,26
plasmic droplet of caput spermatozoa is consistently located in a proximal position near the neck of the middle piece. A few spermatozoa have bent middle pieces associated with translocation of the cytoplasmic droplet and spermatozoal maturation. When spermatozoa reach the proximal cauda epididymidis, most have completed translocation of the cytoplasmic droplet, as noted by droplets located near the junction of the middle piece and the principal piece. Under normal circumstances, few ejaculated spermatozoa have cytoplasmic droplets. Other more subtle, but necessary, changes acquired by spermatozoa during epididymal maturation include remodeling of the surface lipids, proteins, and glycocalyx; reorganization of acrosomal molecules; further changes in the structural stability of the nuclear chromatin; development of signaling pathways; and alterations in spermatozoal metabolism. The collective biochemical changes, both intracellular and extracellular, incurred by spermatozoa during epididymal maturation remain a mystery, but experimentation is gradually revealing new findings.

4. Spermatozoal Structure: Form to Function

The general form of the spermatozoon was first described by Leeuwenhoek 330 yr ago (1677). General acceptance of his proposal that this tadpole-shaped form contributed to fertilization required ~200 years. Thereafter, the structure of the spermatozoon received mounting attention. Resolving power of early microscopes hampered the ability to provide an exquisite description of spermatozoal anatomy, but introduction of electron microscopes in the middle of the 20th century paved the way for intricate viewing of the spermatozoon and its substructure. Excellent descriptions of spermatozoal ultrastructure were provided by Saacke and Almquist in 1964, and by Fawcett in 1975. More recently, Eddy provided a exceptional review of the topic, and included molecular-related insights that have been acquired in recent years.

The spermatozoon is typically divided anatomically into a head and a flagellum (or tail). The head contains the nucleus, overlying acrosome, and a reduced complement of cytosolic elements. The head can be subdivided into an acrosomal region, equatorial segment, post-acrosomal region, and posterior ring, which demarcates the junction between the head and flagellum. The posterior ring is the site of plasma membrane anchoring to the nuclear envelope and is thought to produce a tight seal that separates cytosolic components of the head and flagellum. The flagellum can be subdivided into a connecting piece, middle piece (or midpiece), principal piece, and end piece (Figs. 29 and 30). These various parts of the spermatozoon are surrounded by a common plasma membrane, although the composition of the plasma membrane can be subdivided into regional domains that impact its multiple functions, such as sperm-oviductal adhesion, penetration of the cumulus-
The equine spermatozoon has similar general structures to that described for the bull, ram, boar, dog, and human. The average length of an equine spermatozoon is reported to be 61–86 μm, with the average lengths of the head, midpiece, principal piece, and end piece reported as 5.8–7.0, 9.8–10.5, 43.8–67, and 2.79 μm, respectively. The maximum width of the head is reported to be 2.9–3.9 μm. The size of an equine spermatozoon, relative to its body size, is remarkably smaller than that of some other species, such as the mouse, rat, hamster, and honey possum, where spermatozoal...
lengths are 123, 190, 189, and 356 μm, respectively.\(^{113}\) For further comparison, the average length of a human spermatozoon is 57 μm, with an average head length of ~4.5 μm.\(^ {113}\)

The equine spermatozoon head is defined as splatulate-shaped, in contrast to the falciform-shaped sperm heads characteristic of some species (e.g., the mouse, rat, and hamster). Of the laboratory animal species, the equine spermatozoon most closely resembles that of the rabbit. The spermatozoal head is somewhat elliptical in shape, is flattened in one plane (dorsally) and is thicker in the posterior portion of the head than in the apical portion of the head (Figs. 30 and 31).\(^ {14,19,26}\) The nucleus, which occupies the majority of space within the spermatozoon head, contains the paternal genetic material. Specifically, the male genome is compromised of the X or Y chromosome and a haploid number of somatic chromosomes. This genetic material is packaged for delivery to the oocyte for fertilization, where two haploid (male and female) genomes are combined to produce a diploid offspring. The chromatin (i.e., the DNA and associated proteins) within the nucleus of the mature spermatozoon is highly condensed, resulting in a volume that may approach only 5–10% of that of a somatic cell. This packing is a result of a marked alteration in the composition of nucleoproteins that occurs during epididymal transit. The haploid genome of the round spermatid encodes for unique spermatozoal proteins, termed protamines, which predominate as nucleoproteins during spermatzoal maturation in the epididymis. The cysteine residues of this protein establish intramolecular and intermolecular disulfide linkages that result in compaction and stabilization of the associated DNA. This design is thought to provide protection to the chromosomes during their perilous journey within the female reproductive tract and to streamline the spermatozoon to enhance its mobility on activation at the time of ejaculation.

The nuclear envelope, consisting of a double membrane (each with a lipid bilayer), separates the contents of the nucleus from the surrounding cytoplasm. Although the nuclear envelope is regularly perforated by nuclear pore complexes in somatic cells, such pores are absent over most of the spermatozoal nuclear envelope, i.e., in the area under the acrosome and in the post-acrosomal region. The exception is the region of the redundant nuclear envelope, a portion of the nuclear envelope posterior to the chromatin that folds and extends back into the neck region. This portion of the nuclear envelope contains abundant hexagonally arranged pores. The caudal portion of the nuclear envelope forms a concavity, termed the implantation fossa, which is the site of attachment with the flagellum. This region of the nuclear envelope is overlain with a thick sheet of material, termed the basal plate.
The acrosome is a membrane-bound exocytotic organelle that overlies the rostral two thirds of the nucleus, with a fit resembling that of a bathing cap. Anatomically, the membrane is subdivided into an inner acrosomal membrane that is continuous with an outer acrosomal membrane. These connecting

* A spermatogonia may be found in all eight stages (I-VIII)
** B1 spermatogonia may be found in stages IV and V

Fig. 17. Bright-field micrographs depicting the eight stages of the cycle of the seminiferous epithelium in paraffin sections stained with PAS and toluidine blue. Stage I, characterized by two generations of leptotene (L) and pachytene (P) primary spermatocytes. Sb1 round spermatids can be observed with a well-developed and easily recognizable acrosomal cap. Developing tails are sometimes visible, although these structures can be difficult to distinguish in paraffin sections. Stage II, two generations of L and P primary spermatocytes. Sb2 spermatids become irregularly shaped as elongation and orientation begins. Acrosomal caps are plainly evident. Stage III, two generations of zygotene (Z) and pachytene spermatocytes are evident, as are characteristic Sc spermatids which are oriented with acrosomal caps toward the basement membrane. No primary spermatocytes with meiotic figures are evident. Stage IV contains the most germ cell types of any state. B1 spermatogonia are sometimes evident (data not shown) along the basement membrane next to zygotene primary spermatocytes. In the adluminal compartment, large pachytene spermatocytes begin their divisions and are seen with numerous meiotic figures (MFs; metaphases I and II). Secondary spermatocytes (SSs), newly formed Sa round spermatids, and Sc elongated spermatids are also evident. Stage V, B1 spermatogonia are still evident. Only one generation of pachytene primary spermatocytes may be found. The newly formed Sa round spermatids have not yet begun formation of the acrosomal cap over the nucleus. Sd1 elongated spermatids with densely packed chromatin may be found stacked in clusters, deeply embedded within the seminiferous epithelium. Stage VI: small, round B2 spermatogonia with small clumps of heterochromatin have formed and one generation of pachytene spermatocytes may be found near the basement membrane. Sa round spermatids may be observed with an acrosomic vesicle not yet touching the nucleus. Sd1 elongated spermatids begin their migration toward the lumen. Stage VII, B2 spermatogonia and one generation of pachytene primary spermatocytes may be found. Acrosomal caps of Sa round spermatids begin to flatten and cover the surface of the nucleus. Densely packed Sd2 elongated spermatids are migrating closer to the lumen. Stage VIII, B2 spermatogonia have divided to form small round preleptotene (Pl) primary spermatocytes along the basement membrane. One generation of pachytene primary spermatocytes and late Sa round spermatids with developing acrosomal caps can be found. Residual bodies (Rb) accumulate as mature spermatzoa are being released into the lumen as spermiation occurs. Scale bar = 30 μm.
membranes enclose a narrow heterogenous compartment, termed the acrosomal matrix. The inner acrosomal membrane is closely apposed to the nuclear envelope whereas the outer acrosomal membrane underlies the plasma membrane. These two membranes converge at the level of the equatorial segment. The acrosome originates from the Golgi apparatus in round spermatids during spermiogenesis. Light microscopic changes of the developing acrosome can be visualized in cross sections of the seminiferous tubules (Figs. 4 and 13). Refinement in acrosomal morphology and biochemical composition continue as spermatids traverse the epididymis. In the mature spermatozoon, the acrosome contains a bountiful supply of active molecules, both within the acrosomal matrix and as components of the inner acrosomal membrane. These molecules, which include an assortment of protein receptors and hydrolytic enzymes, are thought to be important for adhesion to, and penetration of, the zona pellucida and sperm–oolemma interactions. During the course of the acrosome reaction, the outer acrosomal membrane fuses with the overlying plasma membrane, thereby creating hybrid vesicles and pores that lead to release and exposure of acrosomal contents (Fig. 32). Although the spermatozoal acrosome contains several enzymes characteristic of a typical cellular lysosome (in addition to those enzymes specific to the spermatozoon), the enzymes are not used within the cell as in autophagy for cellular organelle remodeling and renewing, or in heterophagy as occurs in phagocytic cells. The cytosolic compartment of the mature spermatozoal head is virtually free of organelles other than the acrosome, and the mature spermatozoon is considered to be transcriptionally quiescent.

Fig. 18. Bright-field light (A, C, and E) and scanning electron (B, D, and F) micrographs of different stages of the equine spermatogenic cycle. (A and B) Stages I and II are shown. (C and D) Stages III, IV, and V are represented. (E and F) Stages VI and VII reveal changes in preparation of the release of spermatozoa in stage VIII. Scale bar = 20 μm.19
Fig. 19. A clockwise arrangement of eight stages of the cycle of the equine seminiferous epithelium observed by Nomarski optics in unstained, 20-μm Epon histologic sections. Both nuclear and cytoplasmic details are revealed in Leydig cells (LCs), Sertoli cells (SCs), various types of germ cells, and myoid cells (MCs). A spermatogonia (A) of different types and Sertoli cells are found in all stages of the cycle. Classification of spermatid development was based on that described for humans and used in the horse. Briefly, the Sa spermatid is the earliest form of spermatid, as it contains a spherical nucleus and a large Golgi with either no acrosomic vesicle, a developing acrosomic vesicle, or an acrosomal cap. The Sb1 spermatid has a spherical nucleus but also has an attached flagellum and a distinct acrosome covering half of the nuclear surface. The Sb2 spermatid has begun nuclear elongation with the appearance of the manchette. The Sc spermatid has a distinct manchette and a more elongated nucleus than the Sb1 spermatid. The Sd1 spermatid is undergoing final maturation with the disappearance of the manchette and migration of mitochondria around the tail. The Sd2 spermatid is the final form with a large portion of the cell, including the head, projecting into the tubular lumen in Stage VIII. Stage I (I) is characterized by preleptotene or leptotene (L) and pachytene (P) primary spermatocytes as well as Sb1 spermatids (Sb1). These spermatids are characterized by spherical nuclei, attached acrosomal caps (AC), and developing flagellum (F). Stage II (II) is characterized by leptotene primary spermatocytes (L), pachytene primary spermatocytes (P), and Sb2 spermatids (Sb2). The labeled pachytene primary spermatocyte is displaying its large spherical Golgi apparatus. The Sb2 spermatid has an elongating nucleus, manchette (Mn), and annulus (An). Stage III (III) has zygotene (Z) and pachytene (P) primary spermatocytes, and bundles of Sc spermatids (Sc). The Sc spermatid has an elongating and condensing nucleus, distinct manchette (Mn), and annulus (An). Stage IV (IV) is characterized by the presence of zygotene primary spermatocytes (Z), diplotene primary spermatocytes or secondary spermatocytes (SS), meiotic figures (MF), and Sc spermatids (Sc). Stage V (V) is composed of pachytene primary spermatocytes (P); newly formed Sa spermatids (Sa) and Sd1 spermatids (Sd1). The Sa spermatid has a distinct Golgi apparatus (GA), developing acrosomic vesicle (AV), and chromatoid body (CB); and Sd1 spermatids form bundles which are deeply embedded in the seminiferous epithelium. Stage VI (VI) has type B spermatagonia (B), pachytene primary spermatocytes (P), Sa spermatids (Sa) with their grouped mitochondria (GM) and Sd1 spermatids (Sd1) with their annulus (An) and less distinct manchette. Stage VII (VII) is characterized by type B spermatagonia (B), pachytene primary spermatocytes (P), Sa spermatids (Sa), and Sd2 spermatids (Sd2). Sd2 spermatids, the most advanced form, are migrating toward the tubular lumen in this stage. Stage VIII (VIII) has newly formed preleptotene (Pl) and pachytene (P) primary spermatocytes, late Sa spermatids (Sa) with their acrosomic granule (AG) and newly attached developing flagellum, and Sd2 spermatids lining the lumen with their migrated annulus, enlarged middle pieces (MP) and attached cytoplasmic droplet (CD). Residual bodies (RB) left behind by spermiation of spermatids can be seen near the luminal surface and in transit toward the base within Sertoli cell (SC) cytoplasm. Scale bar = 10 μm.
less, the cytoplasm of the head region contains cytoskeletal proteins that are important to spermatzoal function. Structural changes occur in the cytoskeleton (both microfilaments and microtubules) during spermiogenesis that lead to the reshaping of the spermatid head to an elongated form. This appears to be primarily mediated by the acrosome-acroplaxome-manchette complex and the perinuclear theca.120–122 The acroplaxome is formed at the leading edge of the developing acrosome. This structure contains both filamentous actin and keratin, and anchors the developing acrosome to the underlying nuclear envelope. The manchette consists of a circumferential bundle of microtubules that assemble posterior to the developing acrosome at the perinuclear ring, and extend along the developing flagellum to a point where the fibrous sheath begins (Figs. 13 and 14).123 The manchette may aid nuclear elongation, as well as directional migration of molecules necessary for both nuclear condensation and tail formation.120,121 The perinuclear theca is located between the inner acrosomal membrane and the nuclear envelope (i.e., the subacrosomal region), as well as between the nuclear envelope and the plasma membrane posterior to the developing acrosome (i.e., the postacrosomal region). This theca is composed of a complex of highly condensed

Fig. 20. The spermatogenic wave revealed by an enzymatically-isolated equine seminiferous tubule that was fixed in glutaraldehyde, followed by osmium tetroxide, infiltrated with Epon, mounted in toto in Epon, and observed by bright field microscopy. The corresponding drawing reveals the consecutive stages along the length of the tubule as determined by observation with Nomarski optics. Although modulations (reversal of order) occur, adjacent stages are in consecutive order. Latter stages (more developed; higher numbers) are observed in the same direction of two sets of all stages I–VIII. Scale bar = 200 μm.42

Fig. 21. Light microscopic view of a cross-section of an equine seminiferous tubule, showing the nuclei of some spermatogonia that label positive (brown-black) with an immunohistochemical apoptotic detection assay (termed the TUNEL assay). The background tissue stain is PAS-toluidine blue. The germ-cell associations in the cross sectional view are consistent with stage V of the seminiferous epithelial cycle.43
cytoskeletal proteins that have actin-binding properties and seems to be involved in reshaping of the spermatozoal head.\textsuperscript{121} The connecting piece of the flagellum consists primarily of a capitulum, segmented columns of fibers, and the proximal and distal centrioles. The connecting piece serves to attach the flagellum to the head, and also functions to produce and stabilize some structural components of the flagellum. The capitulum articulates with the head at the level of the implantation fossa, attaching by fine filaments that connect the capitulum to the basal plate. The segmented columns anchor the dense fibers of the flagellum (Fig. 31). The centrioles, oriented at right angles to each other, are involved in development of the connecting piece and the axoneme. The proximal centriole remains attached at the implantation fossa, but the distal centriole gives rise to the axoneme during tail development.

The primary structural elements of the flagellum include the axoneme, the outer dense fibers, the fibrous sheath, and the mitochondria (Figs. 29–31 and 33). The midpiece is characterized by the presence of an axoneme, an array of outer dense fibers, and an overlying sheath of helically arranged mitochondria. The midpiece joins the principal piece at the annulus, a point where the mitochondrial sheath is replaced with a fibrous sheath. The principal piece thereby consists of a centralized axoneme, outer dense fibers of variable length, and a fibrous sheath. The fibrous sheath terminates at the junction of the principal piece and end piece, with the end piece consisting of a small extension of the axoneme (or individually arranged acrosomal microtubules) past the termination site of the fibrous sheath. The entire flagellum is enveloped by a plasma membrane.

The axoneme is a cylindrical array of nine doublet microtubules that surround two singlet microtubules (termed the central pair) connected by regularly spaced bridges, thus forming the “9 + 2” configuration that is characteristic of both cilia and flagella throughout the plant and animal kingdoms. The microtubules are composed primarily of the tubulin family of proteins. Each of the nine outer microtubule doublets consists of an “A” subunit, which is completely cylindrical and composed of 13 protofilaments, and a “B” subunit, which is C-shaped and composed of 10–11 protofilaments (Fig. 33). The A subunits serve as an anchor for the outer dynein arms and inner dynein arms that possess ATPase activity and generate the force required for axonemal and thereby, flagellar motion, through an attachment-detachment cycle between A- and B-subunits of adjacent doublets. Filamentous nexin links or nexin arms connect adjacent doublets, and radial spokes connect the axonemal doublets to a helical sheath surrounding the central pair of microtubules. The mechanism of axonemal action is discussed below under spermatozoal motility.

The outer dense fibers course from the connecting piece through the midpiece into the principal piece. These fibrous structures are thought to provide structural support and passive elasticity during flagellar bending. Nine irregularly shaped outer dense fibers occupy sites overlying the nine axonemal doublets along the entire length of the midpiece. The outer dense fibers taper at varying locations within the principal piece. Two of the outer dense fibers terminate in the proximal portion of the principal piece, and the structural support of

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**Fig. 22.** Scanning electron microscopic view of the equine testicular parenchyma, showing (A) the rete testis complex (RTC) housed in the connective tissue of the mediastinum around the central vein (CV) of the equine testis, and (B) a cross support within the complex. (A) Seminiferous tubules (STs) are adjacent to the connective tissue around the rete testis complex. Cross supports (open arrow) are found in the rete testis complex and in rete testis tubules (RTT). (B) The cross supports are comprised of a simple cuboidal epithelium overlying a connective tissue core. Testicular spermatozoa may be attached to this epithelium. Scale bars = 500 (A) and 4.6 μm (B).\textsuperscript{15}
these two fibers is replaced by inward extensions of the fibrous sheath.

The mitochondrial sheath forms near the end of spermiogenesis, i.e., after the axoneme and outer dense fibers are fully formed. The axoneme of the flagellum in the developing spermatid originates from the centriolar apparatus and extends outwardly in the flagellar canal (Fig. 14). This canal is created by
movement of the annulus and attached plasma membrane to the posterior aspect of the nucleus, resulting in an infolding of the plasma membrane toward the posterior aspect of the nucleus. The flagellar canal prevents attachment of the mitochondria to the midpiece of the spermatid until the annulus migrates distally to the junction of the midpiece and principal piece. Coincidently, the mitochondria assume

![Image](image_url)

Fig. 25. Scanning (A) and transmission (B) electron micrographs of the epithelium of the caput epididymidis. Tall columnar cells are lined with stereo cilia (extremely long microvilli; Sc). (A) The cytoplasm (C) and luminal surface of these cells are revealed. (B) Section through the apex of these cells reveals forming vesicles (FVs) in the process of endocytosis. Scale bars = 10 μm (A) and 2 μm (B).

![Image](image_url)

Fig. 26. Scanning electron micrographs of spermatozoa in the caput epididymidis with their proximally located cytoplasmic droplets (CD; A), epithelium of the corpus epididymidis with its tall columnar cells with shorter stereo cilia than found in the caput (B), corpus epididymidis spermatozoa undergoing translocation of the cytoplasmic droplet (C), and epithelium and thick smooth muscle (SM) wall of the proximal cauda epididymidis (D). (C) The corpus epididymidis is the site of droplet translocation from the proximal to the distal position. The translocation process appears to occur by bending (arrow) of the tail around the droplet followed by re-straightening of the tail. Scale bars = 10 μm (A), 45 μm (B), 10 μm (C), and 240 μm (D).
their final position in an end-to-end spiral arrangement around the outer dense fibers of the midpiece. The length of the midpiece, and the number of mitochondrial gyres, varies greatly among mammalian species, but is relatively constant within a given species. The equine spermatozoon contains 40–50 mitochondrial gyres. The order and symmetry of the mitochondria along the midpiece may convey a functional effect, as disruptions in this organization have been associated with reduced fertility of stallions.\textsuperscript{125}

The fibrous sheath extends along the entire length of the principal piece, i.e., from the annulus to the end piece. It is composed of two longitudinally arranged fibrous columns that are bridged by a series of interconnecting, circumferentially arranged fibrous ribs. The fibrous sheath is thought to provide rigid structural support and elasticity to the flagellum, but it also serves an important role as a scaffold for a variety of cell-signaling and metabolic events.

5. **Physiological Considerations**

Spermatozoal Motility

Under natural conditions, regulation of spermatozoal motility occurs at three critical points: epididymal reservoir (cauda epididymis and ductus deferens)—suppression of motility; ejaculation—activation of motility; and oviductal reservoir—hyperactivation of motility. Spermatozoa in the cauda epididymis are intrinsically capable of motility,\textsuperscript{68,126} but do not exhibit motility until released from the epididymis. A threshold level of cyclic adenosine monophosphate (cAMP) is present in spermatozoa within the cauda epididymis.\textsuperscript{127} Specific motility-inhibiting proteins have been identified in rat cauda epididymal fluid that, when removed, allow initiation of motility.\textsuperscript{128,129} A pH-dependent inhibitory factor has been reported in bulls.\textsuperscript{130,131} Although such inhibitory factors may exist, it is possible that sperm motility may simply be suppressed by the acidic pH of the epididymal environment. The pH of bull cauda epididymal fluid is reported to be 5.5, and the cytosolic pH of bull epididymal spermatozoa is reported to be 6.5–6.6.\textsuperscript{130,132} Caudal epididymal fluid of bulls, rams, boars, and stallions does not contain measurable quantities of bicarbonate (\(\text{HCO}_3^-\)),\textsuperscript{133} and \(\text{HCO}_3^-\) is known to be a key effector of spermatozoal motility.\textsuperscript{134,135} Bicarbonate is present at fairly high concentrations in seminal
plasma and may be higher in seminal plasma of stallions than some other mammals studied.\textsuperscript{136} Simple exposure of normal spermatozoa to seminal plasma or physiologic fluids will activate spermatozoal motility that is characterized by a moderate-amplitude and symmetrical flagellar beat leading to a forward propulsive trajectory.\textsuperscript{137,138} This form of spermatozoal movement is defined as activated motility and is to be differentiated from hyperactivated motility discussed below. Activated motility is considered necessary for propelling sperm into the oviductal reservoir.

Environmental cues activate spermatozoal motility though a signal transduction mechanism. Although the signaling pathway(s) of activated spermatozoal motility are not resolved completely, an ever-increasing body of information indicates that HCO$_3^-$, calcium ions (Ca$^{2+}$), and cAMP are key signaling components.\textsuperscript{112,139} Transmembrane movement of HCO$_3^-$ into the spermatozoal cytosol is associated with an increase intracellular pH ([pH]$_i$), leading to regulation of cAMP.\textsuperscript{140,141} Activity of Na,K-ATPase and Na$^+/K^+$ exchangers are also of central importance in regulating [pH]$_i$ and sperm motility.\textsuperscript{142–146} Spermatozoal Na,K-ATPase is important for generation of the Na$^+$ gradient required for ion transport. This Na$^+$ gradient permits the Na$^+/K^+$ exchangers to catalyze the coupled exchange of extracellular Na$^+$ for intracellular H$^+$.\textsuperscript{143} A sperm-specific Na$^+/K^+$ exchanger has been localized to the principal piece and likely plays an important role in regulating [pH]$_i$.\textsuperscript{142,145,145} Activation of motility requires that this exchanger is functional.\textsuperscript{145} It is also likely that a Na$^+/HCO_3^-$ co-transporter plays a role in controlling cytosolic pH (Fig. 34).\textsuperscript{140}

Although increased alkalinization of the spermatozoal cytosol is known to activate membrane Ca$^{2+}$ channels, this may be of primary importance in hyperactivation of spermatozoal motility where increased cytosolic Ca$^{2+}$ is required.\textsuperscript{141,147–152} Spermatozoal motility can be activated and maintained for a short time in Ca$^{2+}$-free media for many species,\textsuperscript{126,138,153} but presence of extracellular Ca$^{2+}$ maximizes spermatozoal motility.\textsuperscript{154–156} The flagellum is known to contain a variety of Ca$^{2+}$ membrane transport channels, including
voltage-gated, cyclic nucleotide–gated, transient receptor potential, \( \text{Ca}^{2+} \) release, and CatSper channels. Although the roles of some of these channels remain unknown, their mere presence suggests that they probably contribute in some manner. Sperm \([\text{Ca}^{2+}]_i\) is also known to be regulated by \( \text{Ca}^{2+}\)-ATPases, \( \text{Na}^+/\text{H}^+ \) exchangers, and \( \text{Ca}^{2+}/\text{H}^+ \) exchangers.
The signaling pathway for activated spermatozoal motility involves the cAMP-dependent protein kinase A (PKA) pathway. Intracellular production of cAMP, as it relates to motility, is catalyzed by soluble adenylyl cyclase (sAC). sAC has been identified in the flagellum of spermatozoa and is required for activated motility and capacitation.

Fig. 31. Further magnified illustrations of Fig. 29, revealing mid-sagittal and partially resected views of equine spermatozoa. (A) Plasma membrane. (B) Acrosome. (C) Outer acrosomal membrane. (D) Inner acrosomal membrane. (E) Nuclear envelope. (F) Post-acrosomal lamina. (G) Nucleus. (H) Basal lamina and underlying capitelum. (I) Proximal centriole. (J) Segmented column. (K) Outer dense fibers. (L) Outer doublets of axoneme. (M) Center pair of microtubules within the axoneme. (N) Mitochondria.

Fig. 32. Transmission electron micrograph revealing sagittal views of adjacent equine spermatozoal heads. One spermatozoon has an intact acrosome (solid arrow) and one has undergone the acrosome reaction induced by the calcium ionophore, A23187 (open arrow).

Fig. 33. Magnified illustrations of cross-sections through the middle piece (midpiece) and principal piece regions of equine spermatozoa. (A) Plasma membrane. (B) Mitochondria. (C) Outer dense fiber. (D) Axonemal doublet. (E) Central pair of axonemal microtubules. (F) Sheath surrounding central pair of axonemal microtubules. (G) Radial arm. (H) Fibrous sheath. (I) Outer dynein arm. (J) Inner dynein arm. (K) Longitudinal column of fibrous sheath. (L) Connecting bridge between central pair of axonemal microtubules. (M) Nexin links. (N) Anulus.
Interestingly, the cAMP-sAC-PKA pathway does not seem to be required for hyperactivated motility or the acrosome reaction. sAC is stimulated directly by HCO$_3^-$ or Ca$^{2+}$, thereby catalyzing phosphorylation of PKA, which leads to phosphorylation of flagellar proteins that are central to activated motility. One such protein is A kinase anchoring protein (AKAP). AKAP has been detected at the level of the axonemal central pair, the fibrous sheath (in the principal piece), and the outer dense fibers (in the middle piece). PKA is known to bind to AKAP, thus suggesting that the action of PKA can be regionalized, and its substrate specificity aided, through this anchoring mechanism. Although PKA is known to be located in the vicinity of the outer dynein arms, the need for a localized pattern of PKA distribution remains unresolved.

In addition to regulating the cAMP-sAC-PKA pathway, Ca$^{2+}$ affects dynein activity through direct control of the central pair of microtubules within the axoneme. As Ca$^{2+}$ can inhibit the activity of the axonemal central pair to regulate dynein arm function, excessive Ca$^{2+}$ can lead to cessation of spermatozoal motility.

Calmodulin (CaM), a Ca$^{2+}$-binding and sensor protein, is a key component of spermatozoal signaling mechanisms. CaM associates with the outer dynein arms and the radial spokes, and may play a role in Ca$^{2+}$-dependant propagation of flagellar bending. CaM kinase II (CaMK) and CaM-dependent protein phosphatase are central to dynein function. One study indicated that CaM can also interact with T-type voltage-gated Ca$^{2+}$ channels by a mechanism independent of PKA, CaMK, or CaM-dependent protein phosphatase. In addition, CaM may be involved in regulation of adenylyl cyclase.

A substantial and continuous supply of energy, in the form of ATP, is required for activated spermatozoal motility, and this requirement is heightened for hyperactivated motility. The mechanisms by which ATP is generated and transferred in the flagellum remain unsolved. Certainly, oxidative respiration within the mitochondria yields a plentiful supply of ATP. Some investigators propose that the ATP produced in this manner is capable of diffusing along the entire length of the flagellum in a manner suitable for initiation and maintenance of spermatozoal motility. Possibly, an adenylate kinase shuttle may assist in the rapid distribution of mitochondrial-produced ATP. Recently, adenylate kinases were regionalized to the principal piece of mice spermatozoa. Others contend that local glycolysis within the principal piece is critical to the timely generation and distribution of ATP required for spermatozoal motility. Certainly, a variety of glycolytic enzymes have been identified in association with the fibrous sheath and/or outer dense fibers and it is well known that the glycolytic pathway to energy production is essential under anaerobic conditions. Arguments in favor of oxidative res-
piration and of glycolysis exist for generation and supply of ATP for spermatozoal motility. A sperm-specific glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has been localized to the principal piece, and targeted deletion of this enzyme is reported to lead to a severe reduction in motility of mouse spermatozoa. In contrast, another study revealed that chemical inhibition of GAPDHs did not impact spermatozoal motility or ATP concentration. At present, it seems possible that either oxidative respiration or glycolysis, or both, can support the ATP generation and availability needed to drive spermatozoal motility. The length of the principal piece in stallions is similar to that in bulls, boars, rams, and dogs. It is also considerably shorter than the principal piece of mice, rats, hamsters, and guinea pigs, where many experimental studies are conducted. This may explain some of the inconsistencies among laboratories.

The axoneme, dynein arms, outer dense fibers, mitochondrial sheath, and fibrous sheath are often portrayed as the fundamental elements of the flagellum. Although each is vital to flagellar function, one must also be aware that these elements are embedded in a network of other molecules that are equally important. Bending of the flagellum is caused by reciprocal sliding between doublet microtubules within the axoneme. The dynein arms are permanently attached to one doublet microtubule with arms extending to intermittently engage an adjacent doublet microtubule. The dynein arms are situated at 24- (outer dynein arms) to 96-nm (inner dynein arms) intervals along the entire length of the axoneme. When the crossbridges created by the dynein arms are complete between neighboring doublets, the corresponding microtubules are prevented from sliding. Dynein is high molecular weight ATPase, so the dynein arms have the capacity to transform chemical energy into unidirectional mechanical force. Each dynein arm has three ATP-sensitive binding sites, and all sites required for microtubule sliding around the circumference, and along the length, of the cylindrical array of microtubules that compose the axoneme. This dynein-generated activity is regulated by the central pair of microtubules and their associated structures, collectively called the central apparatus. The asymmetry of components in the central apparatus forms the basis of a “timing device” that creates the spatially timed sliding of various microtubules. As such, the central apparatus both constrains and activates the dynein arms through communication through the radial spokes.

Spermatozoal Metabolism

Spermatozoa require a constant supply of energy for maintenance of cellular order and functions needed for survival. This energy requirement increases significantly with the onset of activated motility, and becomes even more pronounced when hyperactivated motility is initiated. Approximately 500 common metabolic reactions are known to occur in most somatic cells, many of which require energy. A spermatozon is a “stripped-down” cell that is designed expressly for propagation of genes, and it is one of the smallest cells in the body, but it accomplishes its mission at a distance far from its origin in the epididymis. While spermatozoa require a relatively small amount of energy for “housekeeping” purposes, a plentiful supply of substrate is required to provide a spermatozoon with the stamina required for this arduous journey. Spermatozoa are also capable of prolonged survival both within and outside the body. Exogenously derived nutrients are needed to fulfill the energy demands of these very metabolically active cells. These nutrients (or substrates) are metabolized intracellularly, resulting in the release of chemical bond energy. Useable energy is made available for cellular processes primarily in the form of the activated carrier molecule, ATP, with other macromolecules such as NADH, NADPH, FADH2, and acetyl CoA also providing vital high-energy intracellular linkages.

Spermatozoa possess the metabolic machinery required for glycolysis, the citric acid cycle, and oxidative phosphorylation. Glycolysis occurs in the cytosol and has both an anaerobic and aerobic mode, whereas the citric acid cycle and oxidative phosphorylation are strictly aerobic and proceed within the mitochondria. A variety of substrates can be garnered for energy extraction, including monosaccharides, pyruvic acid, lactic acid, and even fatty acids and amino acids, the last four of which require an aerobic environment. Sorbitol, a sugar alcohol obtained by reduction of glucose through the polyol pathway, is thought to serve as a substrate for metabolism. Although the seminal plasma of
stallions does contain this product, stallion spermatozoa seem to be incapable of metabolizing it to produce ATP. Generally speaking, glycolysis is considered the “backbone” of the energy-procurement pathways because it can proceed in situations of low oxygen tension and its product, pyruvate, can be further catabolized to CO₂ and H₂O by oxidative respiration. Efficiency of energy production is lowest in the glycolysis pathway, where one molecule of a monosaccharide can generate a net increase of two molecules of ATP. Conversely, complete oxidation of glucose through the citric acid cycle and oxidative phosphorylation generates an additional 34 molecules of ATP per monosaccharide molecule. Despite the increased efficiency of oxidative metabolism, a monosaccharide cannot directly enter the citric acid cycle, but must first be metabolized to pyruvate; hence, the need for glycolysis as the first stage of energy production where monosaccharides are concerned.

Glycolysis involves a sequence of 10 separate reactions, the first of which is catalyzed by hexokinase in an ATP-consuming manner. This enzyme can phosphorylate several types of monosaccharides, including glucose, fructose, mannose, and galactose. The affinity of hexokinase for glucose is reported to be 20 times higher than its affinity for fructose. Another account indicated that, in mammalian spermatozoa, glucose is used in preference to fructose, but that mannose has the highest affinity of the three monosaccharides to hexokinase. Of interest, a study involving astroglial cells revealed that mannose-phosphorylating ability is only 40% of glucose-phosphorylating ability.

Fructose was identified as the “seminal sugar” in 1946. Subsequent studies have revealed that the concentration of this sugar is high in seminal plasma of bulls, rams, and man (range of 40–1000 mg/100 ml) but extremely low (<1 mg/100 ml) in seminal plasma of stallions. Interestingly, another report indicated that fructose concentration in stallion semen ranged from 7 to 11 mg/100 ml. Seminal plasma concentrations of some other metabolizable substrates, such as citric acid, lactic acid, and pyruvic acid (used exclusively by oxidative respiration), are also much lower in stallion seminal plasma than that of bulls, rams, or man. Bull and ram spermatozoa use fructose at a rate of 2 mg/10⁹ spermatozoa/h under anaerobic conditions at 37°C, whereas boar spermatozoa use fructose at a rate that is 10 times lower. One report suggests that stallion spermatozoa have a limited capacity to use fructose. Interestingly, fructose is not found in seminal plasma of dogs and glucose is used preferentially to fructose by dog spermatozoa; yet, fructose maintains higher spermatozoal motility than does glucose under in vitro storage conditions. Heterogeneity may also exist among motile dog spermatozoa, with subpopulations experiencing increased responsiveness to both fructose and glucose.

Either glucose or fructose can increase motility and ATP concentration of human spermatozoa. One study with human semen revealed that spermatozoal motility was maximized in medium containing 1 mM glucose or 15 mM fructose; however, motility was further enhanced when both monosaccharides were provided. Conversely, in vitro fertilization in mice and rats is not attainable when fructose is the only available substrate but high when glucose is included in the media. Other studies certainly support the importance of glucose in gamete interaction.

Transport of monosaccharides across the plasma membrane requires the presence of carrier proteins and is an ATP-dependent process. One report involving bull spermatozoa indicated that the glucose transport protein has only a limited ability to transport fructose and that another carrier protein may be the primary means by which fructose gains access into the cytosol. This carrier protein may be absent in stallion spermatozoa, thus explaining why fructose may not be a primary, or even a suitable, energy source in this species.

The extent to which spermatozoa use glycolysis or oxidative respiration for generation of energy varies considerably among mammalian species and probably with the profile of spermatozoal motility, and environmental conditions. Few studies have been directed toward stallion spermatozoa so much of the information must be extrapolated from information garnered from other species. This approach may be precarious, however, because of the known species variation in spermatozoal metabolic preferences. One report suggests that bull and ram spermatozoa use the glycolytic pathway at a much higher rate than stallion spermatozoa and that stallion spermatozoa rely more heavily on aerobic metabolism for energy production. Others report that ram and bull spermatozoa oxidize acetic acid in preference to fructose and glucose. Human, boar, and mouse spermatozoa seem to obtain a high proportion of their ATP by glycolysis. Although more intensive study with stallion spermatozoa is required to more fully elucidate metabolic pathway preferences, glycolytic breakdown of glucose is currently considered to be a major source of ATP production.

The minimal oxygen tension required to maintain a linear rate of O₂ uptake by rabbit spermatozoa has been reported to be ~10 mmHg. As such, the luminal environments of the uterus and oviduct are thought to be sufficiently high in oxygen content to allow aerobic respiration to proceed. Storage of spermatozoa outside the body cavity, however, can impact availability of oxygen and metabolic processes. If raw or extended semen is left undisturbed in a laboratory setting, use of dissolved O₂ by aerobic respiration leads to depletion of O₂ and the need to resort to glycolysis for meeting energy
demands.\textsuperscript{214} For oxidative metabolism, bull and ram spermatozoa use O\textsubscript{2} at a rate of \textasciitilde10–20 \(\mu\)l/h/10\textsuperscript{8} spermatozoa at 37°C.\textsuperscript{218} A potential drawback of oxidative respiration is the production of reactive oxygen species that can lead to a disruption of ATP production, lipid peroxidation, or other cellular mechanisms that result in spermatozoal dysfunction.\textsuperscript{242–246}

Metabolism of endogenous substrates by spermatozoa for the production of energy is thought to be minimal, possibly representing 10% or less of total energy production, based on calorimetric and carbon balance techniques.\textsuperscript{247} For bull spermatozoa, endogenous metabolism occurs at a constant rate at storage temperatures between 20°C and 35°C.\textsuperscript{248}

Spermatozoal capability for glycogen synthesis and storage was once thought to be nonexistent. Recent reports, however, indicate that spermatozoa of dogs, boars, rams, and horses do contain glycogen, as well as the enzymatic machinery required for its production.\textsuperscript{192,249,250}

The pentose phosphate pathway does not seem to be directly involved with spermatozoal motility,\textsuperscript{218} but may be central to sperm–oocyte interactions,\textsuperscript{251} possibly through production of NADPH and the resultant generation of radical oxygen species required for sperm–oocyte fusion.\textsuperscript{252} Furthermore, the pentose phosphate pathway may aid in protection against oxidative injury through generation of NADPH, which is required to return expended glutathione peroxidase to its reduced (protective) form (see Reactive Oxygen Species).\textsuperscript{253,254}

### Reactive Oxygen Species

A discussion directed at reactive oxygen species (ROS) at this point in the text seems appropriate because of the pathologic consequences that ROS can have on spermatozoal motility and spermatozoal metabolism. Reactive oxygen species are products derived from the reduction of (i.e., addition of electrons to) diatomic oxygen (O\textsubscript{2}) and include radicals and other reactive products. Radicals are atomic or molecular species that have unpaired electrons in their orbits, thus making them quite unstable, i.e., highly reactive and likely to participate in a variety of chemical reactions to displace, receive, or share electrons so that they may once again become stable.\textsuperscript{255} Other O\textsubscript{2} derivatives are not radicals but are highly reactive; hence, these products are collectively termed ROS. Examples of ROS (where * implies the presence of an unpaired electron) include superoxide radical (O\textsubscript{2}•*), hydroxyl radical (OH•*), hydroperoxyl radical (HO\textsubscript{2}•*), peroxy radical (RO\textsubscript{2}•*), alkoxyl radical (RO•*), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and subclasses of ROS that contain reactive nitrogen or chlorine species, such as nitric oxide (NO•*), peroxynitrite (ONOO•−; where NO\textsuperscript{−} + O\textsubscript{2}− •* → ONOO•−), or hypochlorous acid (HOCl).\textsuperscript{255} ROS may assume physiologic roles, as discussed under Capacitation; however, their presence can also lead to spermatozoal demise.\textsuperscript{56,256–260}

Spermatozoa are exposed to ROS that are derived both intracellularly and extracellularly. Production of superoxide occurs continuously within the mitochondrial electron transport chain, where O\textsubscript{2} acts as an electron carrier during oxidative respiration. A molecule of O\textsubscript{2} must pick up a total of four electrons to form water (i.e., \(O_2 + 4 H^+ + 4 e^- \rightarrow 2H_2O\)). During this process, the reactive oxygen forms are generally caged within the respiratory enzyme complexes. A small percentage of the superoxide produced does not stay within the mitochondrial respiratory chain but escapes to exert actions on other cellular components, including lipids, sugars, proteins, and nucleic acids.\textsuperscript{255,258,261–264}

Recently, presumed non-mitochondrial production of O\textsubscript{2}•* has been shown to occur in spermatozoa of both men\textsuperscript{265} and stallions.\textsuperscript{266} Evidence is mounting that spermatozoa contain NADPH oxidase that catalyzes the formation of superoxide, presumably for physiologic reasons,\textsuperscript{267–270} although some workers conclude that spermatozoal-derived NADPH oxidase activity is insignificant.\textsuperscript{254,271,272} An aromatic amino acid oxidase, released from dead spermatozoa or present in seminal plasma, has also been reported to generate production of H\textsubscript{2}O\textsubscript{2} production in semen.\textsuperscript{273–276}

Generation of ROS is known to be more pronounced in morphologically abnormal spermatozoa, a feature that can be associated with retention of excessive amounts of residual cytoplasm.\textsuperscript{258,277–261} Damaged or morphologically abnormal equine spermatozoa are known to generate more ROS than do morphologically normal sperm.\textsuperscript{292}

Neutrophils are known to be a primary exogenous source of ROS in semen of men, where a relatively high concentration of leukocytes is commonplace.\textsuperscript{283,284} Although spiking equine semen with activated neutrophils can lead to reduced motility,\textsuperscript{285} contamination of equine semen with sufficient neutrophils to affect spermatozoal motility is quite uncommon.

Another source of ROS is the uterine environment, such as the ROS generated from estrogen-induced uterine NADPH oxidase of the endometrial epithelium.\textsuperscript{276,286} Cycle-regulated synthesis of uterine NO has also been described.\textsuperscript{287,288} Leukocytes of mares produce H\textsubscript{2}O\textsubscript{2} in response to spermatozoa or bacteria, and this activity is heightened by leukocyte exposure to seminal plasma,\textsuperscript{289} suggesting a means for uterine clearance of bacteria and spermatozoa. Others, however, report a protective effect of seminal plasma against ROS,\textsuperscript{280,290,291} suggesting that seminal plasma of some stallions is known to possess reduced antioxidant capacity.\textsuperscript{292,293}

Unsaturated lipids are quite susceptible to peroxidative injury when exposed to various ROS, and mammalian spermatozoa are especially susceptible because their plasma membranes are rich in polyunsaturated fatty acids.\textsuperscript{242,245,257,294–296} A recent report indicates that the spermatozoa of individual men vary in their membranous unsaturated fatty
acid content and that a superabundance of polyunsaturated fatty acids predisposes spermatozoa to oxidative injury. Peroxidative injury has been thought to adversely affect membrane fluidity, although some work suggests that the oxidative action may not occur through lipid peroxidation. Oxidation may directly affect proteins and membrane permeabilization (possibly through oxidation of sulphhydryl groups on enzymes and membrane proteins), as opposed to disturbing lipid fluidity. Others have described an effect of ROS on the sperm axoneme and ATP generation that can lead to an irreversible loss of motility.

In stallion spermatozoa, a ROS-associated reduction in motility was not associated with a detectable increase in lipid peroxidation or a decrease in viability or mitochondrial membrane potential. This finding suggests that ROS-related effects on equine spermatozoa may occur through a mechanism unrelated to lipid peroxidation and also indicates that motility may be a more sensitive indicator of ROS-related injury than the other experimental endpoints, i.e., viability, mitochondrial membrane potential, and lipid peroxidation. Others report that lipid peroxidation is a good predictor of sperm motility and consider a lipid peroxidation assay to be a potential clinical test.

The mechanisms of ROS-induced damage to DNA have been evaluated intently. Sperm DNA is known to be susceptible to oxidative injury, resulting in reduced fertility and perhaps even pregnancy loss or a variety of pathologic entities in offspring. Aberrant DNA repair by spermatozoa is thought to increase the mutagenic load of any resulting conceptus, thereby leading to post-fertilization crises. Mitochondrial DNA is more susceptible to H$_2$O$_2$-induced damage than is nuclear DNA, possibly making it a good marker of oxidative injury. Spermatozoa of stallions are susceptible to ROS-induced DNA fragmentation.

Protection against the effects of ROS in spermatozoa is afforded by an assortment of scavenging molecules, including three enzyme systems: (1) superoxide dismutase (SOD), (2) catalase (CAT), and (3) the glutathione peroxidase system (GPX), as indicated by the following reactions:

$$\text{O}_2^{-\cdot} + \text{O}_2^{-\cdot} + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2$$  \hspace{1cm} (1)

$$2 \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2 \text{H}_2\text{O} + \text{O}_2$$  \hspace{1cm} (2)

$$2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}$$  \hspace{1cm} (3)

Glutathione reductase (GR) reduces GSSG to GSH to complete the cycle as follows:

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GRD}} 2 \text{GSH} + \text{NADP}^+$$

Superoxide dismutase is considered to be prevalent in spermatozoa of many species and rapidly reduces O$_2^{-\cdot}$ to H$_2$O$_2$. The direct cellular action of O$_2^{-\cdot}$ is thought to be minimal, with its major effects produced indirectly through conversion to H$_2$O$_2$. Spermatozoa of stallions are thought to have limited intrinsic SOD, with SOD activity derived primarily from the seminal plasma through adsorption to the plasma membrane.

Glutathione peroxidase has also been identified in spermatozoa or seminal plasma and is considered to impart protection against oxidative injury incurred by H$_2$O$_2$, however, a primary action of glutathione may be protection against peroxy radicals of polyunsaturated fatty acids (RO$_2^{-\cdot}$) through conversion to relatively inert hydroxyl fatty acids. Glutathione also plays a structural role, as shown by sulfhydryl oxidation and cross-linking associated with nuclear condensation and assembly of the midpiece. Glutathione is present in stallion semen but the source in semen seems to be primarily of seminal plasma origin.

Spermatozoa from most species contain little to no catalase; however, seminal plasma is generally high in catalase activity. The amount of catalase in rabbit semen is genetically influenced (estimated heritability value of 0.48). Stallion semen contains catalase activity that is primarily derived from prostatic secretions. Several studies have shown that addition of catalase to semen of various species will help neutralize the detrimental effects of H$_2$O$_2$ on spermatozoa after in vitro storage. Similarly, oviductal fluids may contain catalase in sufficient quantity to protect sperm from oxidative injury. Surprisingly, stallion semen with higher initial activity of CAT, SOD, and GSH did not lead to improved retention of spermatozoal motility or membrane integrity after cooled storage. Addition of CAT to extended equine semen did not improve maintenance of motility after cooled storage. Similarly, addition of CAT, SOD, or GSH, ascorbic acid, or α-tocopherol to semen extender did not improve post-thaw quality of stallion spermatozoa. Studies with ram spermatozoa suggest that CAT, SOD, and GSH are ineffective at preventing acute peroxidative injury to lipids and incorporation of CAT in extenders for sex-sorted or non-sorted semen does not improve post-thaw quality of ram spermatozoa.
Overall, spermatozoa have limited endogenous CAT, SOD, and GPX activity; therefore, spermatozoa depend on extracellular availability of these enzymes to counter oxidative stress, namely from seminal plasma. Seminal plasma contains these enzymes, in addition to a host of other free radical scavengers.\(^{276}\) Molecules with antioxidant activity include, but are not limited to, ascorbic acid,\(^{336,337}\) α-tocopherol,\(^{246,334,336,338–340}\) hypotaurine,\(^{342}\) butylated hydroxyanisole (BHA; an antioxidant used for long-term preservation of food, cosmetics, and pharmaceuticals),\(^{246,334,339}\) albumin,\(^{261,342,343}\) cysteine,\(^{344,345}\) lipoic acid,\(^{346–348}\) xanthurenic acid,\(^{349–351}\) carnitine,\(^{352,353}\) ergothioneine,\(^{354}\) and pyruvate.\(^{349,355}\) Many of these have been used as dietary supplements, or as direct semen treatments, with mixed results.\(^{296,299,332,340,356–358}\)

As the end product of glycolysis, pyruvate serves as an important substrate for oxidative phosphorylation, but when present at relatively high concentrations, pyruvate can also exhibit antioxidant properties.\(^{355,359}\) Addition of pyruvate to milk-based extender at a concentration of 2 mM is reported to improve equine spermatozoal motility after cooled storage.\(^{349}\)

Presently, there is much controversy over types, doses, and delivery methods for antioxidants as they relate to semen processing. Further study is required to determine whether antioxidant therapy will maximize spermatozoal function, especially after cooled or frozen storage.

**Spermatozoal Transport**

Spermatozoa are virtually immotile in the epididymis but develop motility (or, more precisely, activated motility) on ejaculation. Deposition of spermatozoa is intrauterine when artificial insemination is used, and a large portion of an ejaculate is deposited directly into the uterine body at the time of natural coitus if the mare’s cervix is dilated at the time of breeding and if the stallion does not dismount prematurely during the ejaculatory process. After intrauterine deposition of semen, the spermatozoa are rapidly transported to the oviduct, where a spermatozoal reservoir forms and the spermatozoa gain fertilizing potential before interaction with the vestments of the oocyte near the ampullar–isthmic junction.

Spermatozoal migration to the oviducts is dependent, to a large part, on uterine contractions.\(^{360}\) The effect of insemination volume on the frequency of these uterine contractions seems variable,\(^{361,362}\) but, within the range tested (5–50 \(\times 10^6\) spermatozoa/ml), spermatozoal concentration had no apparent effect on spermatozoal numbers recovered from mare oviducts at 4 hours after insemination.\(^{363}\)

The time required for spermatozoa to gain access into the oviduct has not been studied extensively. Spermatozoa have been detected in the oviducts as early as 2 h after insemination, based on recovery of spermatozoa in abattoir specimens.\(^{364}\) Sufficient spermatozoa to establish pregnancy may be transported into the oviduct as early as 30 min after insemination, based on extensive lavage of the uterus post-insemination with an iodine-based solution to immobilize intrauterine spermatozoa. Pregnancy rates are maximized by delaying the uterine lavage until 4 h after insemination.\(^{365}\) Location of intrauterine insemination seems to have a significant impact on the spermatozoal number that gains access into the oviduct. In one study, a higher number of spermatozoa were recovered from the ipsilateral oviduct after deep uterine-horn insemination than resulted from uterine body insemination. In the same study, the number of oviductal spermatozoa recovered from reproductively normal mares and mares susceptible to post-mating endometritis were similar after artificial insemination of 500 million total spermatozoa.\(^{366}\) Others have found more spermatozoa, and a higher percentage of motile spermatozoa, in the isthmic oviductal region of reproductively normal mares than in mares susceptible to chronic uterine infection.\(^{367}\) The same investigators reported more spermatozoa in the oviducts when mares received semen from a fertile stallion as opposed to a subfertile stallion.\(^{367}\) Another group reported an effect of stallion on spermatozoal numbers in recovered oviducts of mares after artificial insemination, although the fertility or semen quality of these stallions was not provided.\(^{368}\)

Administration of oxytocin to mares immediately after insemination did not improve pregnancy rates in mares bred by either fertile or subfertile stallions.\(^{369}\) This may be because of a directional pattern of luminal flow toward the cervix after oxytocin administration, as occurs naturally during the expulsive stage of labor (i.e., uterine evacuation).\(^{370,371}\) It is interesting to note, however, that dynamic scintigraphic analysis of radiolabeled spermatozoa in the mare uterus revealed radioactivity in the tips of the uterine horns as early as 8 min after uterine body insemination (5-ml volume).\(^{360}\) In this study, uterine contractions did not propagate spermatozoa in one direction only; rather, movement continued in both tubal and cervical directions. Similarly, ultrasonographic evaluation of the uterus after natural mating of mares revealed similar patterns of uterine contractions in cervico-tubular and tubo-cervical directions.\(^{362}\) The peristaltic contractions were increased in frequency, amplitude, and duration in comparison with the pre-coital findings. It seems that bidirectional myogenic activity in the early period after insemination assists spermatozoal transport to the utero-tubal junction, and it is possible that this mechanism is impeded by administration of supraphysiologic doses of oxytocin or other drugs with oxytocic properties. Studies in pigs support this line of thought, because intrauterine infusion of cloprostenol before insemination reduced oviductal recovery rates of spermatozoa and increased cervical reflux of inseminates.\(^{372}\) In another report involving pigs, spiking a semen dose with 10 IU oxytocin reduced fertilization rate, whereas IV administr-
tion of the same dose at 5 min after insemination improved fertilization rate. The addition of another uterotonie agent, prostaglandin F2α, to extended semen (final concentration of 125 μg/ml) did not improve the pregnancy rate in a small group of mares.

The involvement of other factors that may impact spermatozoal transport in horses requires further examination. As an example, studies involving pigs revealed that the mere presence of a boar (i.e., non-tactile presence) could increase peripheral plasma oxytocin concentration in the sow, and uterine activity after boar exposure was most enhanced in sows that initially had a reduced frequency of uterine contractions. Similar studies involving horses are contradictory, with some reports of increased myometrial activity and plasma oxytocin concentrations after simple stallion exposure, and another report indicating no increase in uterine contractions until mechanical stimulation of the vagina and cervix occurs.

It seems logical that seminal plasma contributes to uterine transport of spermatoza after natural cover, because it provides a medium to aid peristaltic movement of spermatoza toward the oviducts. Mann et al. showed the presence of two chemical markers of seminal plasma in the uterine horns at 50 min after mating, with concentrations similar to those detected in fresh ejaculates. These data are consistent with the concept that most of the post-coital fluid in the uterus is derived from the seminal plasma. Using the same chemical markers, these scientists also showed that seminal plasma may also gain access into the oviducts. The physiologic significance of seminal plasma in spermatozoal transport extends beyond that of a simple vehicle. Seminal plasma contains a rich assortment of both organic and inorganic constituents. Prostaglandins have been identified in relatively high concentration in the seminal plasma of humans, monkeys, and the great apes, whereas the concentration in seminal plasma of stallions is minuscule. Of interest, the seminal plasma of boars contains an appreciable concentration of estrogens (up to 12 μg per ejaculate), and this steroid is thought to stimulate myometrial contractions by inducing the release of PGF2α from the endometrium. Spiking boar semen with estradiol-17β, however, did not improve pregnancy rate or fetal number after artificial insemination of gilts. We are unaware of reports relating to estrogens in seminal plasma of stallions.

Seminal plasma is a well-known modulator of spermatozoan-induced uterine inflammation, a feature that likely plays an integral role in removal of spermatoza from the uterus. Troedsson et al. reported that some proteins in equine seminal plasma can protect viable, but not damaged, spermatoza from binding to neutrophils. Although this finding may not impact spermatozoal transport at the first breeding of an estrous cycle, it could affect spermatozoal transport mechanisms if a second insemination were placed in an inflamed uterus induced by the first insemination. Furthermore, it could potentially impact semen transport in mares bred for the first time in an estrous cycle if the mare had a pre-existing acute form of endometritis. To that effect, pregnancy rates in mares were dramatically increased on the second insemination of an estrous cycle (i.e., 12 h after insemination of 1 × 10⁹ killed spermatozoa in semen extender or infusion of semen extender only to induce an endometritis) when the inseminate contained washed spermatoza in seminal plasma (17/22; 77%) compared to washed spermatozoa in semen extender only (1/22; 5%). Clement et al. reported embryo-recovery statistics for mares inseminated two to three times in an estrous cycle (at 2-day intervals) until ovulation occurred, using different stallions for each insemination. For mares inseminated twice in an estrous cycle, 14 of 17 embryos recovered resulted from the first insemination (82%), whereas when mares were inseminated three times in an estrous cycle, only 1 of 6 embryos recovered resulted from the first insemination (17%). Therefore, the first insemination was not uniformly the most fertile. This study was not designed to evaluate the effect of seminal plasma on pregnancy rates, and separation of insemination times by 48 h may have resulted in a uterine environment that was not as hostile as that reported by Troedsson et al. Seminal plasma concentration of inseminates was not reported in this study but is presumed to be >5%. In a study reported by Metcalf, mares were inseminated with frozen-thawed semen from two different stallions during an estrous cycle at a 4- to 10-h interval, followed by parentage determination of the resulting foals. Of nine foals born, four were the result of the first insemination (44%) and five were the result of the second insemination (56%). Although the second inseminate likely contained some seminal plasma, the amount would have been relatively low if the semen was processed for freezing in a standardized manner. The seminal plasma concentration of the frozen semen was not provided in the report. Based on the combined data from these three reports, it would appear that seminal plasma does provide some protective effect for spermatozoa entering an inflamed uterine environment, but the seminal plasma concentration in the inseminate can be quite low and still achieve this effect.

Regardless of the method(s) used to promote uterine transport of equine spermatozoa, in the end, only a small fraction of inseminated sperm reach the oviductal luminae. In one report, only 0.0006–0.0007% of inseminated sperm was recovered from oviductal flushings of mares at 18 h after intrauterine insemination. Barring oviductal occlusion, deposition of spermatozoa in the uterine body of mares leads to similar spermatozoal numbers entering either oviduct. This finding suggests that chemotactic effects are not present to attract uterine spermato-
Spermatozoa can persist in the mare for up to 6 days before ovulation, yet result in establishment of pregnancy. The mechanism of spermatozoal attachment to the oviductal epithelial cells has received considerable study and seems to consist of a specific sperm–ligand interaction involving glycoconjugates (i.e., lectin-like molecules on the surface of spermatozoa interacting with carbohydrate-containing moieties on the surface of oviducal epithelial cells). Recently, galactose-binding proteins were characterized on equine spermatozoa that may prove to be involved in the sperm–oviduct binding mechanism.

This intimate oviductal cell contact with spermatozoa in the oviductal reservoir seems to play two divergent roles: assisting with the final maturation events of spermatozoa that must occur before fertilization of an oocyte, and also maintaining spermatozoa in a viable quiescent state to allow for an extended storage period. Binding of equine spermatozoa to oviductal epithelial cells under in-vitro culture conditions results in both a quantitative and a qualitative change in protein synthesis and secretion by the epithelial cells.

Purified oviducal glycoprotein and polypeptides secreted by oviductal epithelial cells have been shown to have positive effect on spermatozoal capacitation, including sperm–oocyte interactions. Peripheral proteins of the oviductal membrane seem to contribute to maintenance of boar spermatozoal viability. Stallion spermatozoa that are bound to oviductal epithelial cells in culture exhibit flagellar motion for up to 4 days, with gradual release of spermatozoa during that time frame. The oviductal support system and gradual spermatozoal-release pattern are consistent with the concept of ensuring that appropriately prepared spermatozoa are available to “greet” an ovulated oocyte soon after its arrival in the oviduct.

Spermatozoa attached to the isthmic epithelium will subsequently detach and traverse the oviduct to the vicinity of an oocyte. The mechanism for detachment is speculative but likely involves acquisition of hyperactivated spermatozoal motility to break the connection with the oviductal epithelial cells. The lectin-like molecules on the spermatozoal surface responsible for specific binding with the oviductal cells may also be released during the capacitation process, thereby assisting with spermatozoal detachment. Detection of spermatozoa probably yields cells that are both hypermotile and primed for spermatozoon-oocyte interaction.

Spermatozoal Capacitation

In mammals, freshly ejaculated spermatozoa are not immediately capable of fertilizing an oocyte. Early studies showed that spermatozoa require residence time in the female reproductive tract to gain this capability, later termed capacitation. Subsequent studies have revealed that similar changes are required by spermatozoa subjected to fertilization by in vitro methods (i.e., conventional in vitro fertilization, IVF). The process of spermatozoal capacitation includes several discernable events that occur in the oviduct after spermatozoan arrival, including the acquisition of hyperactivated motility, the alteration of sperm surface charge, and the modulation of surface receptors relevant to sperm–oocyte interactions.
vitro fertilization (IVF)), as reviewed by Yanagimachi. As such, capacitation is considered to be an absolute requirement of mammalian spermatozoa destined to fertilize an oocyte without mechanical assistance (i.e., by intracytoplasmic sperm injection [ICSI]). The process involves a series of preparative biochemical and biophysical changes in the spermatozoon that prime it to respond to signals originating from the oocyte and its surrounding cumulus. If capacitation is incomplete, spermatozoa are unable to penetrate the cumulus matrix, and have greatly reduced ability to penetrate the zona pellucida, undergo a zona-induced acrosome reaction, and fuse with the oolema.

Two early laboratory hallmarks for verifying completion of capacitation were acquisition of a hyperactivated pattern of motility and inducibility of the acrosome reaction (Fig. 35). Although capacitation has been defined by some as the changes that render a spermatozoon capable of undergoing the acrosome reaction, spermatozoa are also known to exhibit a hyperactivated state of motility when exposed to capacitating conditions. The term was originally defined as the physiological changes of the spermatozoon in the female genital tract before they are capable of penetrating and fertilizing eggs; thus, hyperactivated motility and the acrosome reaction would both be considered components of capacitation, based on its original meaning. Even though the acrosome reaction and hyperactivated motility are important for fertilization, the kinetic and temporal relationships of these two features are not well understood. Interestingly, human spermatozoa that undergo hyperactivated motility are predominantly cells with normal morphology. In recent years, a variety of molecular markers have been identified that allow more critical evaluation of the molecular events that emerge during capacitation (Fig. 35). Unfortunately, many of these assays can be technically challenging or have not become feasible for clinical use.

Neither the precise signaling mechanisms that govern the attainment of capacitation, nor the exact cellular characteristics of a capacitated spermatozoon, are fully elucidated despite considerable study in this area over the past few decades. Nonetheless, some key signaling pathways and cellular events have been identified and include surface (membrane) alterations, as shown in the diagram.
cytoskeletal modifications, influx and efflux of cytosolic constituents, and a myriad of enzymatic processes. The reader is directed to several reviews on this topic.

Interestingly, mature spermatozoa (i.e., those in the caudae epididymis or in ejaculated semen) seem to be pre-programmed to undergo capacitation, and the process can be induced by a variety of signals, as opposed to a specific molecular event. This is exemplified by the fact that in vitro culture of spermatozoa in many different media types can evoke capacitation. It seems that nature has provided spermatozoa with a broad array of capacitation induction alternatives to ensure that they may retain fertilizing potential even if some evokers are rendered nonfunctional. Figure 36 provides the reader with some of the proposed signal transduction pathways for spermatozoal capacitation. Although the literature is replete with potential activation mechanisms, in our view, those presented in this figure represent an accurate reflection of the most widely accepted signaling pathways. Species differences are known to exist in these regulatory pathways, even among eutherian mammals. Few in-depth studies have been conducted with stallion spermatozoa, so much of our predictions must be extrapolated from studies performed with other species. Conclusions drawn from work in non-equids may be misleading and thereby require verification through experiments involving stallion spermatozoa. It is also important to point out that ejaculates contain a heterogenous population of spermatozoa, and the cells do not undergo capacitation in a highly synchronous fashion. An advantage gained by such an arrangement is the continuous replenishment of capacitating spermatozoa available in the oviduct to await exposure to an ovulated oocyte, because spermatozoal lifespan is shortened appreciably by the capacitation process.

Spermatozoa are bathed in epididymal and ejaculatory fluids that contain “decapacitation factors” that become adsorbed to the surface of the plasma membrane. Although these factors and their regulatory roles have not been fully elucidated, they aid in suppressing premature initiation of the capacitation process during spermatozoal passage to the isthmic portion of the oviduct. Desorption of these extracellular factors may unmask some key signaling mechanisms for capacitation. A cysteine-rich protein family, termed CRISP, associate with spermatozoa in the epididymis as well as through the seminal plasma. These proteins have been shown to inhibit tyrosine phosphorylation and capacitation of spermatozoa, possibly through their ability to block ion channels within the plasma membrane.

Alterations in membrane-associated cholesterol, and cytosolic concentrations of bicarbonate and calcium ions ([HCO₃⁻], and [Ca²⁺], respectively) seem to be central evokers of capacitation in spermatozoa studied under in vitro conditions and the same likely applies in vivo. Efflux of cholesterol from the plasma membrane requires the presence of sterol acceptors in the milieu, such as albumin or lipoproteins. Under laboratory conditions, bovine serum albumin or β-methyl cyclodextrin are generally used to achieve this effect. The net result of cholesterol efflux seems to be destabilization and increased fluidity of the plasma membrane, combined with externalization of key surface receptors. The cholesterol efflux is hypothesized to result in an increased permeability of the membrane to HCO₃⁻ and Ca²⁺. Both of these ions are thought to enter the cytosol from

Fig. 36. A schematic model of some documented and hypothesized molecular events associated with spermatozoal capacitation. Please refer to the text for an explanation of these signaling pathways and for figure abbreviations.
the extracellular space through ion-specific channels. A variety of channels, transporters, and stores exist in spermatozoa that aid in regulation of 

\[ \text{Ca}^{2+} \]. Voltage-dependent \text{Ca}^{2+} channels (VDCCs) exist in the plasma membrane of spermatozoa, and membrane depolarization mediates \text{Ca}^{2+} entry through these channels. Additionally, an increase in cytosolic pH (pH\text{\textsubscript{i}}) markedly increases the activity of VDCC. Internalization of HCO\text{\textsubscript{3}}\textsuperscript{−} has been shown to further enhance \text{Ca}^{2+} entry through a PKA-dependent mechanism. Interestingly, \text{Ca}^{2+}-P\text{\textsubscript{i}} symporters and Na\text{\textsuperscript{+}}-HCO\text{\textsubscript{3}}\textsuperscript{−} co-transporters are also known to exist in mammalian spermatozoal membranes and are thought to be involved in the capacitation process. Plasma membrane \text{Ca}^{2+}-ATPase (PMCA), an enzyme pump that extrudes \text{Ca}^{2+}, is activated by the presence of decapacitation factors. In their absence, PMCA activity is decreased, resulting in a net increase in \text{Ca}^{2+}. A family of transmembrane \text{Ca}^{2+}-selective ion channels, called CatSpers, has been identified in recent years. These channels are thought to play an important role in both activated (non-capacitated) and hyperactivated (capacitated) motility. CatSper2 channels have been localized to the flagellum, and CatSper1 channels have been detected in the principal piece of the flagellum.

Receptors for adenosine, calcitonin, and fertilization promoting peptide (seminal plasma constituents) have been identified in the plasma membrane of spermatozoa and seem to play a modulating role in capacitation through regulation of membrane-associated adenyl cyclase (mAC). These molecules activate mAC in uncapacitated spermatozoa but down-regulate mAC in capacitated cells, possibly to prevent “overcapacitation” of cells. Another method of preventing overcapacitation of spermatozoa is the polymerization of actin. These molecules have been identified in many areas of the spermatozoal membranes and seem to tether PKA and various other signaling enzymes to the flagellum. Actin polymerization is a phos- 

olipase D (PLD)-dependent event, and this enzyme is regulated by both PKA and protein kinase C (PKC). The formation of a filamentous form of actin in the peri-acrosomal space may prevent premature fusion of outer acrosomal and overlying plasma membranes when the properties of these two membranes become more fusogenic during the capacitation process. The actin must depolymerize for the acrosome reaction to occur.

An increase in [HCO\text{\textsubscript{3}}\textsuperscript{−}]\text{\textsubscript{i}} and \text{Ca}^{2+} inactivate a soluble cystolic form of adenyl cyclase (sAC), leading to elevated intracellular concentration of cAMP. This nucleotide subsequently activates cAMP-dependent PKA activity, eventually leading to an up-regulation in protein tyrosine phosphorylation. A large number of proteins are known to be tyrosine phosphorylated during capacitation, but the roles of many remain unclear. Indeed, tyrosine phosphorylation of spermatozoal proteins seems to be crucial to acquisition of a capacitated state. Tyrosine phosphorylation of head proteins occurs during capacitation, but this activity is even more pronounced in the flagellum, thereby suggesting an importance of these proteins in hyperactivation. Two members of a tyrosine-containing family of proteins, termed AKAPs, are localized in the fibrous sheath and seem to tether PKA and various other signaling enzymes of the flagellum. These AKAP members are phosphorylated during capacitation and are thought to provide regional control of signal transduction. Similarly, a calcium-binding protein, CABYR, has been localized to the fibrous sheath and is known to be tyrosine phosphorylated during capacitation. Phospholipid hydroperoxide glutathione peroxidase (PHGPx) has been identified in the mitochondrial capsule of the spermatozoal midpiece, and tyrosine phosphorylation of this enzyme during capacitation may play a role in hyperactivated motility. The phospho- 

tyrosine protein, valosin-containing protein, is known to redistribute from the neck region to the anterior head region and may be involved with the acrosome reaction. Other phosphotyrosine proteins may be involved with recognition of the zona pellucida and sperm–zona binding. Another spermatozoal-specific enzyme, scramblase, may be activated through the PKA-tyrosine phosphorylation pathway, leading to a collapse of the asymmetric bilayer distribution of phospholipids, lipid packing disorders, and phospholipase activation.

Some workers consider the HCO\text{\textsubscript{3}}\textsuperscript{−}-cAMP-PKA-tyrosine phosphorylation (scramble) pathway to precede an efflux of cholesterol from the plasma membrane. Flippases, floppases, and an amphospholipid transporter localized to the acrosomal region of mouse spermatozoa are thought to be important in maintaining the lipid asymmetry of the lipid bilayers before capacitation by establishing higher concentrations of phosphatidylserine and phosphatidylethanolamine in the inner leaflet and higher concentrations of sphingomyelin and phosphatidylcholine in the outer leaflet.

Another probable pathway to tyrosine phosphorylation involves formation of ROS that trigger downstream events that lead to capacitation. The effects of superoxide anion (O\text{\textsuperscript{2−}}) and H\textsubscript{2}O\textsubscript{2} on spermatozoal capacitation were first described 15 yr ago. More recently, NO\textsuperscript{−} has been shown to regulate capacitation. These findings have stimulated intensive studies regarding the physiologic roles of ROS in this process. Current evidence supports the concept that ROS induce the cAMP-mediated tyrosine-phosphorylation cascade, but additional routes of action are also quite likely.

As indicated above, the plasma membrane undergoes considerable remodeling during capacitation,
with an appreciable efflux of sterols and transverse asymmetry of phospholipids. These events increase the fluidity of the membrane, thereby allowing horizontal redistribution of membrane molecules. Recent studies have revealed that lipid rafts participate in this redistribution process. These cholesterol-rich microdomains are laden with signaling molecules that are known to be involved with capacitation and zona binding. The capacitation process can increase the number of membrane rafts, as well as their affinity for zona binding. Actin polymerization may play a role in association/dissociation of the membrane rafts. This has become an intense area of study, as these signal-carrying lipid rafts appear to play an increasingly important role in capacitation and spermatozoon–oocyte interaction.

The list of other potential endogenous signaling mechanisms is extensive, including involvement of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERKs), calmodulin-dependent kinases, protein tyrosine K (PTK), PKC, Na⁺/Ca²⁺ exchangers, Na⁺/K⁺/Ca⁺ channels, and the renin-angiotensin system. This list is not meant to be all inclusive, but to show that capacitation involves an extremely complex series of molecular events. Future studies will more clearly define the precise molecules and mechanisms that control capacitation, including spatial, temporal, and kinetic patterns.

**Acrosome Reaction**

Regulated acrosomal exocytosis is a fundamental element of fertilization and might be a more appropriate defining term than “acrosome reaction.” It must be preceded by a complex series of events, including spermatozoal capacitation, followed by spermatozoon–oocyte recognition, spermatozoon–zona binding, and elicitation of signaling pathways. An excellent review of the acrosome reaction was recently provided by Bailey, and the reader is referred to this source for an in-depth account of the biochemical mechanisms involved in the process. Other resources are also available for those that desire a thorough grasp of the subject. Capacitation primes a spermatozoon for the acrosome reaction through alterations in the membrane lipids; membrane hyperpolarization; relocation, aggregation, and externalization of membrane-signaling molecules; and changes in cytosolic and cytoskeletal components. A trigger, however, is required for induction of the acrosome reaction, and most experimental evidence suggests that the zona pellucida provides the inciting factor. Spermatozoal interaction with the zona seems to involve a precise species-specific receptor–ligand interaction, although the exact identity of the spermatozoal receptor remains elusive. Evidence abounds, however, that designated glycoproteins of the zona pellucida, termed ZP3, are responsible for initiating the signaling cascade(s) that lead(s) to the acrosome reaction. The exact signal transduction pathway(s) for acquisition of the acrosome reaction is (are) not fully understood, but decades of intensive study have yielded much enlightening information on the subject. Figure 37 conceptualizes possible pathways involved. As with capacitation, it is quite possible that more than one reaction cascade can elicit the acrosome reaction.

The zona pellucida (ZP) is an extracellular glycoprotein matrix surrounding the oocyte. In most mammalian species studied, excluding humans, the matrix is composed of only three glycoproteins, typically termed ZP1, ZP2, and
The peptide component of these glycoproteins seems to be well conserved across species, but post-translational modifications (i.e., glycosylation) lead to species heterogeneity and hence species-specific binding affinity. The interactions seem to involve recognition of specific carbohydrate moieties present on the polypeptide backbone of ZP3 by complementary zona-binding proteins of the plasma membrane of the spermatozoon. In the mouse, B1.4-galactosyltransferase I (GalT I) is the putative receptor on the spermatozoal surface for a glycoside ligand of ZP3, the binding of which leads to induction of the acrosome reaction. A complex of spermatozoal surface molecules is likely involved in spermatozoan–zona interaction. Whereas ZP3 can be considered the most likely signaling molecule of the ZP for induction of the acrosome reaction, various experiments also suggest that ZP2 may be involved in the anchoring of acrosome-reacted spermatozoa to the zona. The ZP2 and ZP3 exist as heterodimers in filamentous arrangement cross-linked by ZP1, so it seems a fitting motif for integrated spatio-temporal control so that Ca\(^ {2+} \) rises in 

**ZP3**^609,611,614^ The peptide component of these glycoproteins seems to be well conserved across species, but post-translational modifications (i.e., glycosylation) lead to species heterogeneity and hence species-specific binding affinity. The interactions seem to involve recognition of specific carbohydrate moieties present on the polypeptide backbone of ZP3 by complementary zona-binding proteins of the plasma membrane of the spermatozoon. In the mouse, B1.4-galactosyltransferase I (GalT I) is the putative receptor on the spermatozoal surface for a glycoside ligand of ZP3, the binding of which leads to induction of the acrosome reaction. A complex of spermatozoal surface molecules is likely involved in spermatozoan–zona interaction. Whereas ZP3 can be considered the most likely signaling molecule of the ZP for induction of the acrosome reaction, various experiments also suggest that ZP2 may be involved in the anchoring of acrosome-reacted spermatozoa to the zona. The ZP2 and ZP3 exist as heterodimers in filamentous arrangement cross-linked by ZP1, so it seems a fitting motif for integrated spatio-temporal control so that Ca\(^ {2+} \) rises in the plasma membrane, probably transient receptor potential (TRP) channels, thus creating the sustained rise in intracellular Ca\(^ {2+} \) that is required to drive the acrosome reaction. The mechanism of TRP channel activation is unresolved, but both depletion of Ca\(^ {2+} \) from internal stores and direct receptor activation have been proposed methods of activation. The TRP channels have been expressed in both the head and flagellar regions of spermatozoa, suggesting that they may play a role in both the acrosome reaction and in hyperactivated spermatozoal motility. ^640^ Receptor-activated Gi proteins seem to play a central role in the acrosome reaction, as shown by their activation by ZP3–GalT-I binding and their inactivation by pertussis toxin (which inactivates Gi proteins). Activation of Gi proteins leads to a rise in [Ca\(^ {2+} \)], directly evokes Ca\(^ {2+} \) influx through low-voltage-activated calcium channel (T-type channel) activation in membranes. Hyperpolarization of the membrane potential, as occurs during capacitation, is thought to release the channels from inactivation in order that they may respond effectively to a stimulus derived from the zona pellucida, i.e., membrane potential changes are translated into intracellular Ca\(^ {2+} \) signals. A ZP-mediated increase in membrane adenyl cyclase (mAC) has also been reported to occur through Gi protein activation, presumably resulting in the increase in cAMP that is known to occur after spermatozoal exposure to the ZP. Evidence for a cAMP/PKA-mediated acrosome reaction exists after Ca\(^ {2+} \) entry into the cell, but little is known about the role of this pathway in the acrosome reaction. Breitbart and coworkers suggested that PKA may activate a voltage-dependent channel in the outer acrosomal membrane that leads to release of Ca\(^ {2+} \) stores from the acrosome. Zona-induced acrosomal exocytosis can be blocked by an inhibitor of PKA. ^551^ The phospholipase (PLC) family seems to be central to the events leading to the acrosome reaction, and these enzymes have been localized to the spermatozoon head region. Activation of PLC requires Ca\(^ {2+} \), but at relatively low concentrations, as might be produced by the pathways in the preceding paragraph. Zona-mediated co-activation of membrane-bound PLCB1 (through a Gi protein–coupled receptor) and membrane-bound PLC\( \gamma \) (through a tyrosine kinase [TK] receptor) has been proposed. Activation of PLC is thought to promote the acrosome reaction through a couple of routes. First, PLC activates hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP\(_2\)) within the membrane, resulting in production of two active enzymes: diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP\(_3\)). DAG mediates the activation of PKC, and IP\(_3\) releases Ca\(^ {2+} \) from intracellular stores, i.e., the acrosome. Activation of PLC may also lead to disassembly of F-actin filaments that are formed between the outer acrosomal membrane and overlying plasma membrane during capacitation, presumably to separate these increasingly fusogenic membranes. PIP\(_2\) is purported to inhibit actin-severing proteins. Another member of the PLC family, PLC\( \delta \), plays a central role in the acrosome reaction. This enzyme seems to be important for mobilizing Ca\(^ {2+} \) stores in the acrosome and also activating SOCE in the plasma membrane, thereby creating a sustained increase in...
cytosolic Ca\(^{2+}\) concentration.\(^{484,634,655–659}\) The IP\(_3\) receptor and PLC\(_{64}\) have been localized to the acrosome.\(^{655,657}\) and both probably play a role in TRP-channel regulation of Ca\(^{2+}\) entry into the cell.\(^{635,660–664}\)

The role of PKC, as a signaling pathway in the acrosome reaction, remains somewhat unclear. The PKC activity of spermatzoa is dramatically lower than that detected in some other tissues,\(^{665–667}\) but nonetheless, has been identified in various regions of the spermatzoa.\(^{666,668}\) Certainly, DAG, produced through the activity of PLC, is known to be a potent activator of PKC,\(^{665,669}\) possibly leading to opening of a Ca\(^{2+}\) channel in the plasma membrane.\(^{534,640,650}\) DAG is also a membrane destabilizing molecule that increases membrane fusibility.\(^{451,670,671}\) Experimentation has revealed that phosphorylation of spermatozoa proteins occurs on spermatozoal stimulation by progesterone, a known inducer of the acrosome reaction, and the activity can be mimicked with various activators of PKC and inhibited by various inhibitors of PKC.\(^{572}\) Synaptogagmins are a family of calcium-binding transmembrane proteins purported to be calcium sensors responsible for triggering exocytosis in various cells.\(^{673,674}\) Synaptotagmin VI has been localized to the outer acrosomal membrane of human spermatozoa.\(^{675}\) Recently, PKC-mediated phosphorylation of spermatozoal synaptotagmin VI has been shown.\(^{672}\) These workers proposed a model by which PKC has a regulatory role in synaptotagmin function through phosphorylation of synaptotagmin C2 domains.\(^{672,673}\) hence, PKC may play a vital role in controlling the molecular machinery that mediates membrane fusion (discussed below).

Phospholipase A\(_2\) (PLA\(_2\)) is recognized as an important enzyme for the acrosome reaction. Certainly, indirect evidence suggests that treatment of spermatozoa with PLA\(_2\) inhibitors will impede this event.\(^{572,24}\) Both Ca\(^{2+}\) and DAG (in the presence of Ca\(^{2+}\)) are capable of activating PLA\(_2\).\(^{658,680–683}\) The resulting enzymatic action is deacylation of fatty acids from phospholipids, producing fatty acids (such as arachidonic acid and lyophospholipids). A key target is phosphatidylincholine, yielding lysophosphatidylincholine. This “metabolite” is likely to increase the acrosome reaction, because it is known to trigger the reaction in capacitated spermatozoa.\(^{684–687}\) Exposure of capacitated spermatozoa to solubilized zona results in increased metabolism of phosphatidylincholine to arachidonic acid and lysophosphatidylincholine, resulting in a high proportion of acrosome-reacted cells. The PLA activation is regulated by a signal transduction pathway involving Gi protein and DAG.\(^{682}\) Lysophosphatidylincholine, or similar metabolites of PLA\(_2\) activity, seem to be important to the final stages of CAMP-mediated acrosomal exostosis.\(^{688}\)

Regulated exocytosis, as it relates to membrane fusion at neural synapses, has been studied extensively.\(^{689}\) Expanding these studies to spermatozoa has revealed that a similar mechanism of action occurs between the outer acrosomal and plasma membranes,\(^{672,690–692}\) culminating in release of acrosomal contents and exposure of previously hidden domains on the inner acrosomal membrane. Studies to date indicate that the machinery required for this exocytotic event (which requires the finely regulated merger and fusion of two membranes) includes several core components: (1) soluble N-ethylmalamide-sensitive factor attachment protein receptor (SNARE) proteins; (2) N-ethylmalamide-sensitive factor (NSF); (3) soluble NSF attachment protein (α-SNAP); (4) Rab-GTPase; (5) synaptotagmin; and (6) calcium ions. This basic machinery is considered obligate for acrosomal exocytosis.\(^{690,693}\) Of interest, the SNARE proteins, NSF, and synaptotagmin have recently been identified immunocytochemically in stallion spermatozoa, with predominant staining in the regions of the acrosomal cap and equatorial segment.\(^{694}\)

The SNARE proteins are functionally classified as v-SNAREs and t-SNAREs, and membrane fusion is executed when v-SNAREs on the acrosomal (i.e., vesicle) membrane pair the complementary t-SNAREs on the plasma (i.e., target) membrane to form trans-SNARE complexes or SNAREpins. Both v- and t-SNARE proteins are thought to be secured to their respective membranes by hydrophobic anchors that span both leaflets of the lipid bilayers.\(^{689,695,696}\) NSF and its co-factor, α-SNAP, are cytosolic proteins that act cooperatively to regulate SNARE protein function. The α-SNAPs bind to the SNARE protein so as to correctly position NSF, and are responsible for stimulation of the ATPase activity of NSF that dissociates trans-SNARE complexes.\(^{697–701}\) This is a mechanism for recycling of components of the fusion machinery in other cell types, but would not seem necessary in the acrosome, where the reaction is a one-time event. Recent work with spermatozoa indicate that they also serve essential pre-fusion roles,\(^{691,692}\) probably by inducing conformational changes in the SNARE proteins complexes, i.e., catalyzing disassembly of an inactive cis SNARE complex, to allow re-association as active trans complexes.\(^{691}\) The NSF has also been reported to act in a pre-fusion step in non-spermatozoal cells by catalyzing SNARE protein-induced membrane rearrangement that is more suitable for Ca\(^{2+}\)-mediated fusion.\(^{702}\)

Rabs are a family of proteins that are included in the Ras superfamily of G proteins. They are anchored to the acrosomal membrane, a process augmented by cholesterol efflux,\(^{703}\) and, on activation (through guanosine triphosphate [GTP binding]), can interact with Rab effector molecules on the plasma membrane to form an initial physical link, allowing the v-SNARE and t-SNARE proteins to interact to form a trans-SNARE complex.\(^{689,704,705}\) The assembly of the trans-SNARE complex is thought to generate sufficient energy to overcome the repulsive forces generated by the polar head groups of the two membranes.\(^{689}\) In essence, the
acrosome becomes tethered to the plasma membrane. It is possible that that the filamentous F-actin interspersed between the outer acrosomal and plasma membranes could dampen the repulsive forces.451 Epac, a known guanine nucleotide exchange factor (i.e., catalyzes replacement of GDP with GTP on Rab proteins) is activated by cAMP, resulting in induction of the acrosome reaction706; hence, another signaling cascade enters the mix of possible regulatory mechanisms for the acrosome reaction.

Ca\(^{2+}\)-binding synaptotagmin, also a major Ca\(^{2+}\) sensor, is central to both the spatial and the kinetic elements of the fusion process. Synaptotagmin is known to have direct interaction with t-SNARE and SNAP proteins, a feature augmented by Ca\(^{2+}\), and may actually assist in SNARE complex formation.673,707 This protein is a large transmembrane protein whose cytosolic component is primarily C2 domains, similar to that of pKC isoforms.673 In other cell types, the protein is thought to facilitate docking of secretory vesicles close to Ca\(^{2+}\) channels that might be exposed to high local concentrations of this ion.673,708,709 As mentioned above, studies with spermatozoa indicate that synaptotagmin may also inhibit spontaneous fusion, through regulation by PKC. This control is likely to be driven by [Ca\(^{2+}\)].

Without doubt, Ca\(^{2+}\) is the driving force for the final stages of acrosomal preparedness for reaction, from Rab3 activation through final pore formation.691 It seems that Ca\(^{2+}\) is maintained in locally low concentrations during SNARE complex formation, possibly through synaptotagmin control. When trans SNARE complexes are fully assembled for the final fusion event, synaptotagmin likely triggers release of Ca\(^{2+}\).673,691 Studies involving non-spermatozoal cells reveal that the docking mechanism pulls the pre-fusion membranes to within a distance of 2–3 Å.693,710 Subsequent bridging of adjacent phosphate head groups by Ca\(^{2+}\) allows regional intermembranous displacement of water (i.e., by calcium hydration) so that the resulting anhydrous complex is amenable to lipid mixing.691,707–712 The regions of fusion are restricted by the circular array of SNARE complexes.691,711 Similar mechanisms probably exist for spermatozoa because the basic machinery is similar to that of somatic secretory cells.

As with capacitation, the precise pathways involved in the acrosome reaction are not fully elucidated despite considerable study in this area, and species differences are likely. The potential for involvement of signaling pathways and endogenous molecules, other than those listed above, is exemplified by the potential role of angiotensin II in the acrosome reaction of stallion spermatozoa.593,594

Hyperactivated Motility
A hyperactivated form of motility is required to free the spermatozoa from the oviductal reservoir (through spermatozoal release from the oviductal epithelium and subsequent passage through the mucinous environment of the oviduct) and to penetrate the cumulus matrix and zona pellucida surrounding the oocyte.149,152,441,445 It is of interest that hyperactivated spermatozoa tend to have normal morphologic characteristics.53 The observed in aqueous media, the motility pattern of a hyperactivated spermatozoon is characterized by a high-amplitude, often asymmetric, whiplash flagellar beat pattern, leading to a circular or non-progressive trajectory.149,152,441 Studies have revealed that this flagellar wave form actually improves the progressivity of spermatozoa over that of activated motility when spermatozoa are exposed to viscoelastic conditions such as those that exist in the oviduct.441,713

Activated motility and hyperactivated motility may require different environmental signals. Presumably, a signal for hyperactivated motility is elicited within the oviduct under natural conditions to initiate the event at a time that is conducive to fertilization.441 Although the precise signal(s) for initiation of hyperactivation remain(s) unsolved, it is possible that chemotactic and/or thermotactic factors serve in this capacity.714 It is also possible that spermatozoal exposure to alkalinizing conditions, as exists in the oviduct and above that which initiates activated motility, is the primary initiator of hyperactivated motility.456,152,715,716

The signaling pathway is also dissimilar between activated and hyperactivated forms of motility. Activated motility primarily uses the cAMP-sAC-PKA pathway and requires a relatively low concentration of Ca\(^{2+}\) for phosphorylation of flagellar proteins (Fig. 34). Conversely, hyperactivated motility seems to require an elevated concentration of Ca\(^{2+}\) and no cAMP or sAC.166

The precise mechanisms controlling hyperactivated motility are subject to continued study, but an increasing body of literature suggests that an increase in extracellular pH leads to an increase in [pH]\(_i\), thereby potentiating the action of Ca\(^{2+}\) channels.147,467,718,720 The most likely mechanism for an increase in cytosolic Ca\(^{2+}\) is through CatSper channels and through release from internal stores. Four CatSperm channels (CatSper1–4) have been localized to the principal piece, and they seem to have a physical interaction.716 One report suggests that all four CatSper channels must be operational for hyperactivation to occur;716 however, others provide evidence that this may not be the case.721 CatSper1 may serve as an internal pH sensor, as evidenced by the composition of its N terminus.147,543 The redundant nuclear envelope (RNE) at the base of the flagellum has been identified as a possible internal store for Ca\(^{2+}\). This structure represents the remnants of the nuclear membrane following spermogenesis. The RNE is reported to contain IP\(_3\) receptor-gated Ca\(^{2+}\) channels that are thought to release Ca\(^{2+}\) stores and regulate hyperactivated motility independent of similar channels.
in the acrosome. Because the RNE is located at the base of the flagellum, where flagellar bending is propagated, it seems a logical location for an internal Ca\(^{2+}\) store. A specific activator of PKC has been shown to induce hyperactivated motility in human spermatozoa; thus, a PKC signal transduction pathway may also be important to stimulation of hyperactivated motility. Although Ca\(^{2+}\) is known to affect the bending pattern of the flagellum and added Ca\(^{2+}\) is required for hyperactivated motility, the mechanism by which Ca\(^{2+}\) controls this event remains unknown.

The signaling pathways and cellular events leading to hyperactivated motility and to the acrosome reaction are different, and the events of each can occur independently. Nonetheless, internal alkalization seems to be a common inciter for the two events. The pathways, although not the same, achieve the critical spermatozoal priming required for interaction with the oocyte. Because capacitation was originally defined with this endpoint in mind, it seems appropriate to consider hyperactivated motility as a component of the capacitation process. It has been quite difficult, however, to understand the interplay of the various components of capacitation. Indeed, the various events of the capacitation process are not tightly coupled. Penetration of zona-free hamster eggs by human spermatozoa is maximal at 18 h under in vitro conditions, whereas changes in tyrosine phosphorylation occur after 1–2 h, and hyperactivation is maximal after 3 h of incubation. An uncoupling of the acrosome reaction and hyperactivated spermatozoal motility has also been shown in hamsters and bulls. Indeed, we now know a great deal about the molecular control of various facets of the capacitation process, but many more discoveries are needed to uncover the secrets of the spermatozoon.

Sperm–Oocyte Interaction

Although spermatozoal capacitation is not a site-specific phenomenon and can be induced in a variety of artificial media, the caudal segment of the oviduct seems to be the principle location for spermatozoal capacitation and storage under in vivo conditions. Interactions with an ovulated oocyte, however, require spermatozoal migration to the ampullar region of the oviduct, and only a small percentage of spermatozoa that gain access into the oviduct will eventually arrive at this fertilization site. Such spermatozoa are thought to have achieved full fertilizing potential. The precise mechanisms by which spermatozoa migrate to the ampullar region of the oviduct remain speculative, but contractile movements of the oviduct and hyperactivated spermatozoal motility are thought to play key roles in this migratory phase. Evidence is mounting that chemotactic factors are also important to directional migration of oviductal spermatozoa. Some investigators assert that chemotactic responsiveness is also a part of the capacitation process. Spermatozoa possess specific chemotactic receptors. Olfactory receptors are considered to represent the largest family of genes in the human genome, with up to 1000 members, and odorant receptors have been identified, and genes encoding for olfactory receptors have been expressed, in the testes of mammals.

Oriented migration of spermatozoa to follicular factors has been shown under in vitro conditions, and progesterone is thought to be an important mediator of this event. Follicular fluid is not thought to pass down the oviduct to the caudal isthmic region, however, and migrating capacitated sperm can be detected in the more proximal regions of the oviduct before ovulation. Studies involving oocyte transfer in horses have also shown that fertilization can occur when oocytes are transferred to the oviduct contralateral to an ovary containing a preovulatory follicle or when transferred to oviducts of non-cyclic mares supplemented with exogenous steroids. One working hypothesis is that spermatozoa are transported to the general site of fertilization by chemotaxis and contractions of the oviduct and, when in the direct vicinity of the oocyte, are directed by chemotactants secreted by the cumulus cells. Certainly, studies regarding spermatozoal chemotaxis remain scanty at this juncture, but continued study may divulge a specific role of chemotactants in spermatozoal migration patterns.

Before a spermatozoon can interact directly with the oocyte, it must first negotiate passage through the two sizable extracellular matrices which surround the oocyte, i.e., the cumulus complex and the zona pellucida. An excellent description of the three-dimensional structure of the zona pellucida was recently provided. Acquisition of hyperactivated motility and translocation/exposure of glycosylphosphatidylinositol-anchored surface hyaluronidase (termed PH-20) likely permit penetration of the cumulus matrix. Binding of acrosome-
intact spermatozoa to the zona pellucida through species-specific carbohydrate moieties of the zona pellucida and corresponding receptors on the spermatozoal surface lead to induction of the acrosome reaction. An assortment of spermatozoal surface lectins and glycoenzymes have been shown to have zona-binding ability. Previous studies involving mouse spermatozoa suggest that the binding of the spermatozoal surface receptor, GalT-I, to specific oligosaccharide moieties of ZP3 on the zona pellucida is important to induction of the acrosome reaction. More recent work, however, indicates that other mechanisms may be responsible for initial adhesion of spermatozoa to the zona pellucida. A newly voiced theory is that the zona pellucida acts as a spermatozoal scaffold, with ZP2 acting to orchestrate zona permissibility to zona penetration by a spermatozoon, and initial zona penetration by the spermatozoon activating the acrosome reaction through “mechanosensory” signals. One report involving horses suggests that spermatozoal surface galactosyltransferase (GalT) and zona glycoprotein ZPC (probably similar to ZP3 in the mouse) are not required for spermatozoal binding to the zona pellucida. Acquisition of the acrosome reaction was not an experimental endpoint in this study, so it remains possible that a GalT-ZPC–independent mechanism is responsible for initial adhesion, but that a GalT-ZPC–dependent interaction may be required for the acrosome reaction. Passage through the cumulus may also displace the surface-associated sperm glycoproteins, glycode-
with the spermatozoal membrane, most prominently in the distal corpus and cauda epididymis. CRISP family members have been localized to the equatorial region of capacitated and acrosome-reacted spermatozoa. Interestingly, stallion spermatozoa possess an abundance of CRISP, with largest amounts detected in ejaculated spermatozoa. Three members of the CRISP family have been identified in the testis, epididymis, ampullae, or seminal vesicles of stallions. A portion of CRISP is tightly associated with the spermatozoa and is localized to the post-acrosomal and equatorial regions of the sperm head, as well as the mid-piece. The regionalized distribution of the protein hints to a potential role in spermatozoon-oocyte interaction; however, complementary molecules have not been characterized on the oocyte membrane of any species studied. In addition, the lack of hydrophobic domains in this molecule suggests that it may not be directly involved in membrane fusion events.

The spermatozoal ADAM (A Disintegrin And Metalloprotease) family of proteins (including fertilin [ADAM1], fertilin β [ADAM2], and cyritestin [ADAM5]) have been implicated in spermatozoon-oocyte binding and are known to possess fusogenic potential. Nonetheless, gene disruption data do not corroborate an essential role of these proteins in fertilization. The protein, Izumo, has withstood the spatio-temporal, immunological, and gene-knockout tests required to consider it as a probable spermatozoal candidate in gamete fusion. Treatment of spermatozoa with antibodies to integrins or osteopontin also reduce spermatozoon-oocyte binding and fertilization in vitro. Recently, proteomic analysis of the spermatozoal membrane regions involved in spermatozoon-oocyte interactions has uncovered an abundance of proteins that could be participants, including some previously uncharacterized proteins.

A tetraspanin protein, CD9, has been implicated as a very viable candidate oocyte protein for spermatozoal adhesion and fusion, although additional proteins might also be involved. The microvilli of the oolemma seem to be key to spermatozoon-oocyte fusion, and CD9 is enriched in the microvillar portion of the oolemma. In addition, CD9 may be involved in coordination of microvillar shape and distribution along the oolemma. Membrane lipid molecules are also of fundamental importance to spermatozoon-oocyte fusion. In addition, polymerization of actin filaments may be critical to spermatozoal incorporation into the oocyte cytoplasm, decondensation of the nucleus, and activation of the oocyte block to polyspermy. Entry of the sperm into the ooplasm elicits an initial increase in intracellular Ca$^{2+}$ concentration, followed by repetitive Ca$^{2+}$ oscillations. This signaling mechanism induces exocytosis of oocyte cortical granules (to prevent polyspermy); release of the oocyte from meiotic arrest; pronuclear formation; mediation of genomic union; progression into mitosis with reorganization of both nuclear and cytoskeletal components; and stimulation of oocyte mitochondrial respiration. The factor responsible for this activation is derived from the spermatozoal cytosol, and a spermatozoon-specific PLC is considered to be the most likely candidate molecule. In a manner similar to that of the acrosome reaction, PLC might activate hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP$_2$) within the membrane, resulting in production of inositol 1,4,5-triphosphate (IP$_3$), which, in turn, could mediate release of Ca$^{2+}$ from intracellular (i.e., endoplasmic reticulum) stores.

Internal energy generation is required for spermatozoon-oocyte interactions. In the mouse, spermatozoon-oocyte fusion is inhibited in the absence of glucose. In this species, glucose seems to mediate tyrosine phosphorylation of proteins in mid-piece-specific sites. Sperm entry into the mouse oocyte is characterized by pentose phosphate pathway activity and redox regulation, in addition to glycolysis. Interestingly, oocytes are incapable of metabolizing glucose; thus, pyruvate or cumulus cells (which convert glucose or lactate to pyruvate) must be present in IVF medium.

6. Clinical Considerations: Semen Evaluation

Given the lengthy and detailed nature of the above text, one may wonder about its relevance to the clinical arena. Assisted stallion reproduction spans hundreds of years, with references to the topic dating back to Arabic texts (circa 1300) and scientific reports issued from the late 1700s to the early 1900s by noted authorities such as Spallanzani, Repiquet, Hoffman, and Ivanoff. Advancements were furthered by the works of investigators such as Bielanski, Day, Mann, Merkt, and Nishikawa, and the next generation of dedicated scientists, including Amann, Foote, Hughes, Kenney, Palmer, Pickett, and Tischner. These individuals took the discipline of stallion reproduction to new heights. As seen by the information provided in the previous sections, we continue to garner a deeper understanding of spermatozoon structure and function. Although progress in equine reproduction is curbed by funding limitations, our discipline has gained considerable insights from work conducted in humans, laboratory animals, and food-producing animals. An appreciation of the molecular basis of spermatozoon function, spermatozoon-oviductal interactions, and spermatozoon-oocyte engagement will undoubtedly lead to many practical applications in the clinical front, such as assemblage of a battery of in-depth laboratory tests to assess spermatozoon function, expanded treatment options for subfertile stallions, improved methods for preservation of semen, and heightened applications for assisted reproductive technologies such as conventional in vitro
fertilization and intracytoplasmic sperm injection techniques. The bottom line is that, the more we learn, the more informed we can be in decision making regarding diagnostic and therapeutic strategies as they relate to spermatozoal function and reproductive health in stallions. It becomes incumbent on us, and future clinicians and academicians, to convert these opportunities into practical applications.

Conventional laboratory tests for assessment of spermatozoal quality have included light microscopic evaluation of spermatozoal morphologic characteristics and estimation of spermatozoal motility (including percentages of motile and progressively motile sperm; velocity of spermatozoal movement; and longevity of spermatozoal motility following in vitro storage).\textsuperscript{17,817–820} Although other features of semen quality are also considered, including spermatozoal concentration, semen volume, and presence of blood, urine, or potentially pathogenic bacteria, the basic features of this examination process date back several decades. The value of the examination is predicated, to a large extent, on reliable equipment and personnel with good observational power. Even when these stipulations are in effect, the predictive value of the examination can be limited.\textsuperscript{114,115,125,821–833} The same holds true for spermatozoa of other mammalian species.\textsuperscript{834–837} After viewing the previous sections of this paper, one can appreciate that expanded analysis of equine spermatozoal populations might improve the predictive value of the testing process. In fact, additional tests shown to be of diagnostic value are presently incorporated into the spermatozoal examination process within some reference laboratories today.

One of these tests is the sperm chromatin structure assay (SCSA). This assay, introduced by Evenson et al. in 1980,\textsuperscript{838} has been applied to spermatozoa from a number of species, including horses.\textsuperscript{839–841} The SCSA tests a compartment of spermatozoa that is not monitored by conventional methods, i.e., nuclear chromatin. The SCSA is a flow cytometric procedure that uses the metachromatic fluorochrome, acridine orange, and tests the denaturability of spermatozoal chromatin challenged with acid treatment. The literature provides variable results regarding the relationship of stallion spermatozoal chromatin denaturation to the extent of disulfide bonding within and between protamine molecules,\textsuperscript{842,843} however, chromatin susceptibility to denaturation is correlated with the level of actual DNA strand breaks.\textsuperscript{841} The DNA strand breaks can be associated with a myriad of factors, including idiopathic apoptosis, oxidative stress, heat stress, radiation injury, or protamine deficiency,\textsuperscript{843–850} and may involve double-stranded or single-stranded DNA fragmentation or oxidized nucleosides.\textsuperscript{844,851} Such lesions could create genetically defective spermatozoa, leading to germ-line mutations. Interestingly, spermatozoa affected by such damage may appear normal, based on laboratory parameters such as spermatozoal motility and membrane integrity, but may induce post-fertilization embryonic failure.\textsuperscript{852} Because of the highly condensed nature of the spermatozoal chromatin, mature spermatozoa are known to be transcriptionally inactive,\textsuperscript{35} so it is logical that DNA damage might not be expressed until mitosis occurs at the time of spermatozoon–oocyte fusion. This becomes quite important clinically as it represents a potential noncompensable defect, i.e., affected spermatozoa in an ejaculate may not be impaired for fertilization, so increasing the insemination number will not increase pregnancy rate. Ejaculated spermatozoa are known to retain a cohort of cytoplasmic mRNA and translational ability,\textsuperscript{32,35} so it is also possible that fragmentation of mRNA could have a negative impact on some other spermatozoal functions leading to reduced fertilization potential. Assays other than the SCSA are available to measure spermatozoal DNA fragmentation/chromatin disruption, including a TdT-mediated-DUTP nick end labeling (TUNEL) assay, an in situ nick translation (NT) assay, a sperm chromatin dispersion (SCD) assay, and an electrophoresis-based Comet Assay.\textsuperscript{310,844,845,853–857} Although these assays have not been used to the same extent as the SCSA in the equine arena, they are commonly applied in the human field. An immunofluorescence assay has also been developed for evaluation of human spermatozoal protamine levels,\textsuperscript{856} and a similar assay for equine spermatozoa could have diagnostic value.

Another assay that we now commonly use in our battery of tests is the acrosomal responsiveness assay (ARA). This assay is directed at testing the functionality of the spermatozoal acrosome, i.e., its ability to acrosome react when challenged with a potent inducer of the event, the Ca\textsuperscript{2+} ionophore, A23187. Conventional tests are unable to predict the fertility of a subset of stallions with acrosomal dysfunction, because these stallions present with normal spermatozoal morphology and motility, as well as normal chromatin quality (based on SCSA testing) and normal acrosomal structure (based on fluorescent light microscopy and transmission electron microscopy). Meyers et al.\textsuperscript{858} first reported that the acrosomes of subfertile stallions with poor spermatozoal motility did not react readily in response to progesterone exposure. Fertile stallions averaged a 17% acrosomal reaction rate after 5 h of incubation in capacitating conditions followed by exposure to progesterone, whereas subfertile stallions averaged only a 6% response rate. This study indicated that progesterone was capable of stimulating the acrosome reaction in equine spermatozoa exposed to capacitating conditions, and the response in subfertile stallions was reduced. Others have reported that the plasma membrane of stallion spermatozoa contains progesterone receptors and indicate that this may be a pathway for induction of the acrosome reaction.\textsuperscript{859} In this regard, the percent-
age of spermatozoa with exposed progesterone receptors has been shown to be highly correlated with fertility of stallions.660

Other workers identified a subset of subfertile stallions whose only distinguishing spermatzoal characteristic was the drastically reduced ability to acrosome react when exposed to A23187 for up to 3 h (i.e., mean reaction rate of 84% in fertile stallions versus mean reaction rate of 6% in subfertile stallions),661 thereby suggesting the spermatzoal defect may be isolated to the acrosome. Measurement of the cholesterol–phospholipid ratio in semen of these stallions revealed that the ratio was significantly greater in both seminal plasma and whole sperm from the subfertile stallions compared with fertile stallions.660 Further studies are required to dissect the underlying etiology of this condition and to determine appropriate treatment strategies. A keen understanding of the molecular pathways of capacitation and the acrosome reaction, as discussed above, provides us with logical investigative approaches.

Although transmission electron microscopy remains a decisive means for detecting the acrosome reaction in stallion spermatozoa,663 acrosomal status can be assessed by bright-field microscopy using Comassie blue stain.665,666 Fluorescence-tagged lectins or acidotropic probes can be used with light microscopy or flow cytometric methods to assess the acrosome reaction or acrosomal damage.858,866–873 Although the more popular fluoresceinated lectins or acidotropic probes have generally replaced chlortetracycline-based assays for detecting the acrosome reaction or acrosomal damage,858,866–873 the latter assay remains useful because of its ability to gage capacitation, as well as the acrosome reaction.663,874 Another marker shown to be useful for detection of capacitation in stallion spermatozoa is merocyanine 540, an impermeant lipophilic probe that permits evaluation of the architecture and disorder of lipids in the outer leaflet of the plasma membrane bilayer.455,481,513,560 Other probes, such as Annexin V or Rh-damine 123, have largely been replaced by other markers because of its reduced photostability and its tendency to lose retention in the mitochondria following a loss in membrane potential. Some of the MitoTracker probes do not fluoresce until oxidized, so these markers permit easy assessment of the respiratory state of the mitochondria. The JC-1 may be optimally suited for evaluation of the energetic state of mitochondria because it produces a membrane potential-sensitive shift in color pattern, i.e., the color changes from green to red-orange as membrane polarization increases. This is because of the reversible formation of red-orange J-aggregates in a state of high membrane potential compared with green monomeric JC-1 in a state of low to medium membrane potential.873,878–881

The semi-permeable plasma membrane, which is composed of a wide assortment of lipids, proteins, and carbohydrates, envelopes the entire spermatozoon. This structure is vital to regulation of spermatzoal functions by establishing ion gradients, facilitating cytosolic entry of larger molecules and orchestrating various cell-signaling events, to name a few. Thus, assessment of its integrity would seem an important component of a semen evaluation. To this end, a variety of laboratory procedures have been used to evaluate the integrity of the plasma membrane. One method is to evaluate the ability of spermatozoa to exclude extracellular dyes, such as eosin Y, which are nonpermeable when the membrane is intact. Another approach is to expose spermatozoa to hypotonic media (50- to 100-mOsm range) to test their osmolyte or swelling test (HOST).882,883
With this assay, membrane intact spermatozoa theoretically permit excessive water entry into the cytosol, resulting in a variety of morphological changes in the flagellum associated with the cytosolic swelling. Conversely, osmoregulation-incompetent spermatozoa will not experience noticeable changes in flagellar shape. While these tests can serve an adjunctive role in semen evaluation, they are not widely applied in the clinical setting because of the potential for misinterpretation. For instance, humidity impacts the cellular permeability of eosin Y.884 The incidence of spermatozoa with a bent flagellum prior to HOST will also confound the interpretation of results after exposure of a spermatozoal population to hypo-osmotic media.

A broad array of membrane-impermeable fluorescent dyes is available commercially and can be used to test membrane intactness. Examples include the DNA dyes, propidium iodide, bis-benzimide (Hoechst 33258), YO-PRO-1, TO-TO-1, and ethidium homodimer-1. As an alternative, spermatozoa can be bathed in cell-permeable probes that become hydrolyzed to form membrane-impermeant fluorescent products in the cytosol. Examples include carboxyfluorescein diacetate (hydrolyzed by nonspecific cytosolic esterases to form carboxyfluorescein); calcein AM or dihydrocalcein AM (hydrolyzed by esterases to form calcein, which, in turn, forms fluorescent complexes with Ca^{2+}, and other metals), and SYBR-14 (deacetylated in the cytosol, with the resulting product expressing strong fluorescence when complexed with nucleic acids). Of interest, staining of porcine spermatozoa with SYBR-14, which complexes with DNA, does not affect their ability to fertilize oocytes.885 Certain membrane-impermeable and membrane-permeable dyes can be combined in solution before spermatozoal exposure in an effort to provide a more accurate reflection of membrane integrity. For instance, a combination of SYBR-14 and propidium iodide yields three populations of stained spermatozoa: (1) membrane-intact, SYBR-14-stained cells (green); (2) membrane-damaged, propidium-iodide-stained cells (red); and (3) moribund cells (double-stained).886,887 In one study, the percentage of motile stallion spermatozoa (based on computerized motility analysis) was highly correlated ($r = 0.98$) with the percentage of spermatozoa with intact plasma membranes, based on staining with SYBR-14 and propidium iodide.888 Some fluorescent plasma-membrane dyes can also be combined with certain mitochondrial dyes888,889 or acrosomal dyes890–894 to provide more thorough compartmental coverage in the assay. The literature reports triple-stain fluorescent techniques for use with stallion spermatozoa: propidium iodide/SYBR-14/ JC1;888 and propidium iodide/FITC-PNA (an acrosomal lectin probe)/carboxy-SNARF-1 (an intracellular pH indicator).890,895 Although images can be ascertained with the various fluorophores described above by using fluorescence microscopy, flow cytometry is typically applied because of the high throughput and objectivity associated with this approach.870,871,881,894,896 Use of a fluorescence microplate reader assay is also reported for use with JC-1.897

Considerable effort has been directed toward identification of biochemical markers of spermatozoal function that might aid in laboratory-based detection of fertility by targeting specific subcellular compartments or domains. Many of these methods have been devised for use with non-equine subjects, but several have been proposed for potential use with stallion spermatozoa. Although the value of such tests requires further scrutiny and standardization, Table 1 provides examples of assays that may prove valuable as diagnostic tools:

Incorporation of in-depth tests, such as those above, for semen evaluation does not replace or diminish the value of the classical measurements of spermatozoal motility or morphology, and these two methods are likely to remain the hallmarks for semen evaluation. Evaluation of spermatozoal motility in both raw and extended forms is considered to be a fundamental laboratory test for assessing the fertilizing capacity of spermatozoa in an ejaculate. Evaluation of raw (undiluted or neat) semen gives one an idea of how well spermatozoa perform in their natural fluid milieu. Determining motility in the raw form can be hampered by higher sperm concentrations and spermatozoal agglutination to the cover glass, making it difficult for the evaluator to discern individual motility patterns. To overcome this limitation, an aliquot of semen can also be appropriately diluted (e.g., to $25 \times 10^6$ sperm/ml) in a good-quality semen extender that is free of debris when visualized microscopically. The extender may slightly alter motility pattern, usually by increasing the velocity measures. After initial extenion, a high percentage of spermatozoa may exhibit a circular motility pattern; however, this behavior usually resolves after 5–10 min of exposure time in the extender. Spermatozoal motility is exceptionally susceptible to environmental conditions (such as excessive heat or cold, lubricants, light, disinfectants, and osmolality/pH of semen extender), so it is necessary to protect the semen from injurious agents or conditions before analysis. To enhance the reliability of motility estimation, the procedure should be performed by an experienced person using a properly equipped microscope, i.e., one with a built-in stage warmer and properly adjusted phase-contrast optics. Estimations of the percentages of motile and progressively motile spermatozoa are generally determined, in addition to an estimation of spermatozoal velocity (based on an arbitrary scale of 0 [stationary] to 4 [fast]). Subjective assessment of motility is generally quite acceptable, provided personnel are experienced in analysis of spermatozoal motility.

Several different techniques and instruments have been developed in an effort to secure an objective (i.e., unbiased) evaluation of spermatozoal mo-
ility; however, these methods (e.g., time-lapse photomicrography, frame-by-frame playback videomicrography, spectophotometry, or computerized analysis) are generally considered to be too tedious or expensive for routine use. Computerized systems are currently in place in many reference laboratories with the intent to objectively assess motion characteristics of spermatozoa. Despite the commercial availability of various generations of computer-assisted spermatozoal analysis (CASA) systems for >20 yr, their presence has not provided the definitive assay for measuring spermatozoal fertilizing potential. Such an expectation, however, is unrealistic given the numerous independent spermatozoal attributes that are required for a spermatozoon to possess fertilization competence. What these systems do provide is the prospect of objective measurement and protocol standardization. These instruments permit customized selection of various features, including frequency and length of frame capture; threshold demarcations for presence of spermatozoal motion, progressivity of motion, and velocity measures; and gating freedom for both size and luminosity representative of spermatozoal heads, as a means to maximize capture of spermatozoa while minimizing capture of non-spermatozoal material in the sample of interest. Such manipulations are important for improving measurement accuracy and repeatability within a given laboratory, but make it virtually impossible for reliable comparisons among laboratories or among different CASA brands. Computerized analysis of spermatozoa is primarily reserved for the research setting, where standardization, accuracy, and precision are a prerequisite to measurement of experimental endpoints. A distinct value of CASA instruments in the commercial environment (at a veterinary hospital or an equine breeding operation) is the ability to garner objective results for a variety of motility variables. Confusion arises, however, regarding the relationship of the myriad of obtainable CASA variables to fertility of the sample. As an example, we recently conducted a fertility trial with a subfertile stallion whose semen was subjected to density-gradient centrifugation in an effort to improve semen quality before insemination. Values for percent motility (MOT), percent progressive motility (PMOT), and mean curvilinear velocity (VCL; m/s) before, and after, semen processing for the subfertile stallion and a fertile control stallion are listed in Table 2. Based on these results, it would seem that semen treatment for the subfertile stallion yielded a spermatozoal population with quality similar to, or exceeding (based on velocity values), that of the fertile control stallion. Nonetheless, when fertile mares were inseminated hysteroscopically with 20 × 10^6 progressively motile spermatozoa (100-μl volume), the resulting pregnancy rates were 15/20 (75%) for

### Table 1. Biochemical Markers for Assessing Spermatozoal Function

<table>
<thead>
<tr>
<th>Biochemical Marker</th>
<th>Potential Value of Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrosin/amidase activity</td>
<td>Acrosome integrity</td>
</tr>
<tr>
<td>SNARE proteins</td>
<td>Acrosome reaction</td>
</tr>
<tr>
<td>Caspases</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Heparin-binding proteins</td>
<td>Capacitation</td>
</tr>
<tr>
<td>Protein phosphotyrosine activity</td>
<td>Capacitation</td>
</tr>
<tr>
<td>Superoxide anion</td>
<td>Capacitation</td>
</tr>
<tr>
<td>Chromomycin A₃</td>
<td>Chromatin packing</td>
</tr>
<tr>
<td>Acetylcarnitine/carnitine</td>
<td>Motility/morphology</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>Oocyte activation</td>
</tr>
<tr>
<td>C₁₇-BODIPY(581/591)</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>Oxidative injury</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>Spermatozoal motility</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>Spermatozoal maturity</td>
</tr>
<tr>
<td>Creatine kinase/heat shock protein A²</td>
<td>Sperm–oocyte binding</td>
</tr>
<tr>
<td>CRISP proteins</td>
<td>Sperm–oocyte interaction</td>
</tr>
<tr>
<td>SP20/hyaluronidase</td>
<td>Sperm–zona binding</td>
</tr>
<tr>
<td>AWN spermadhesion protein</td>
<td>Subfertile Before 63 48 251</td>
</tr>
<tr>
<td>P34H protein</td>
<td>Subfertile After 90 79 259</td>
</tr>
<tr>
<td>Zonadhesin</td>
<td>Spermatozoal fertility</td>
</tr>
<tr>
<td>SP22 protein</td>
<td>Sperm–zona binding</td>
</tr>
</tbody>
</table>

### Table 2. Effect of Equine Spermatozoal Centrifugation Through a Silanized Silica-Particle Solution on Spermatozoal Motion Characteristics

<table>
<thead>
<tr>
<th>Stallion</th>
<th>Treatment</th>
<th>MOT (%)</th>
<th>PMOT (%)</th>
<th>VCL (μm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertile</td>
<td>Before</td>
<td>80</td>
<td>63</td>
<td>205</td>
</tr>
<tr>
<td>Fertile</td>
<td>After</td>
<td>91</td>
<td>78</td>
<td>209</td>
</tr>
<tr>
<td>Subfertile</td>
<td>Before</td>
<td>63</td>
<td>48</td>
<td>251</td>
</tr>
<tr>
<td>Subfertile</td>
<td>After</td>
<td>90</td>
<td>79</td>
<td>259</td>
</tr>
</tbody>
</table>

MOT = total motility; PMOT = progressive motility; VCL = curvilinear velocity.
the fertile stallion compared with 7/20 (35%) for the subfertile stallion (p = 0.01). This showed that spermatozoal motility does not provide absolute discrimination power, again emphasizing that spermatozoal attributes other than motility play critical roles in spermatozoal fertilizing ability. Some CASA systems can be outfitted with fluorescence optics, and the predictive value of CASA might be improved by incorporation of fluorescent dyes in the media such that motility variables can be segregated by presence or absence of membrane intactness. Further studies are required to determine if the predictive value of spermatozoal motility can be improved by this approach. A study involving boars suggests that CASA analysis might have an improved relationship with fertility if the spermatozoa are also incubated under capacitating conditions to observe changes in spermatozoal velocity over time.

The morphology or structure of spermatozoa is typically examined with a light microscope at ×1000 magnification. Standard bright-field microscope optics can be used to examine air-dried semen smears, provided appropriate stains are used in slide preparation. Specific sperm stains include those developed by Williams and Casarett. General purpose cellular stains (e.g., Wright’s, Giemsa, hematoxylin-eosin) also have been used to accent both germinal and somatic cells in semen smears. Background stains (e.g., eosin-nigrosin, India ink) probably are the most widely used stains because of their ease of application. Visualization of the structural detail of spermatozoa can be greatly enhanced by fixing the cells in buffered formal saline or a similar fixative and viewing the unstained cells as a wet mount with either phase-contrast or, preferably, DIC microscopy. In addition, the incidence of artifactual changes is reduced in comparison with stained smears.

At least 100 spermatozoa should be evaluated for evidence of morphological defects. The type and incidence of each defect should be recorded. Abnormalities in spermatozoal morphology traditionally have been classified as primary, secondary, or tertiary. Primary abnormalities are considered to be associated with a defect in spermatogenesis and, therefore, are of testicular origin. Secondary abnormalities are created in the excurrent duct system. Tertiary abnormalities, as opposed to the previous two types, develop in vitro as a result of improper semen collection or handling procedures.

The current trend is to record the numbers of specific morphologic defects, such as knobbled acrosomes, proximal protoplasmic droplets, swollen midpieces, and coiled tails. This method of classification is considered superior to the traditional system because it reveals more specific information regarding a population of sperm while avoiding erroneous assumptions about the origin of these defects. The origin of some spermatozoal morphologic defects is unknown. Additionally, some morphologic abnormalities like detached heads can be primary, secondary or tertiary in nature, thereby introducing the possibility of error when using the traditional classification system exclusively. The reader is directed to Figure 39 for drawings of normal and abnormal sperm morphologic features, as might be viewed when using DIC microscopy for analysis.

The percentage of morphologically normal spermatozoa is positively correlated with spermatozoal motility. Moreover, close morphological inspection provides additional information about characteristics of individual spermatozoa. This information is important because semen can possess good spermatozoal motility, yet have a relatively high incidence of spermatozoal morphologic abnormalities. Furthermore, stallions can have many spermatozoal abnormalities, yet display normal fertility. Some morphologic defects (e.g., cytoplasmic droplets, bent tails) seem to have a minor effect on fertility of stallions bred by natural cover, whereas other defects (e.g., detached heads, abnormally shaped heads, abnormally shaped midpieces, coiled tails, and premature germ cells) have a deleterious effect on fertility. In general, morphologically abnormal spermatozoa do not exert a direct negative influence on normal spermatozoa. Therefore, the total number of morphologically normal sperm in ejaculates may provide more information regarding the fertility of a stallion than the percentage or absolute number of morphologically abnormal spermatozoa. Generally speaking, the percentage of morphologically normal sperm in a semen sample is similar to the percentage of progressively motile spermatozoa. If spermatozoal motility is low and the percentage of morphologically normal sperm is high, it suggests that laboratory errors occurred that led to a lowering of spermatozoal motility. One cannot discount, however, a potentially negative effect of seminal plasma on spermatozoal motion characteristics.

As seen in the previous paragraphs, a variety of techniques and protocols are available for evaluation of the spermatozoon. Application of newly developed assays can be cumbersome and expensive. Nonetheless, incorporation of some of these diagnostic tools into a standard semen analysis may yield improved discrimination ability regarding the competence of these complex, yet intriguing, cells. Similar applications would be valuable for critical evaluation of laboratory techniques applied to liquid preservation or cryopreservation of spermatozoa.

7. Concluding Remarks

Although a plethora of scientific information surrounds spermatozoal structure and function, many unresolved issues remain, even with human and rodent spermatozoa where the largest body of information has been assimilated. Only when we know precisely the specific molecular interactions required for attaining full spermatozoal fertilizing po-
Fig. 39. Drawings of spermatozoa to resemble images of normal and abnormal equine spermatozoal morphology, as viewed by differential-interference contrast (DIC) microscopy of a fixed and unstained wet-mount semen specimen. The DIC optics provide a three-dimensional image of spermatozoa. 

(A) Normal spermatozoal morphology, in dorsoventral (A1 and A2) and side (A3) views. Abaxial midpieces (A1) are considered to be morphologically normal. 

(B) Variations in abnormal head morphology, including macrocephalic (large head) morphology (B1), microcephalic (small head) morphology (B2), nuclear vacuoles (or crater defects; considered by our laboratory to be normal if low in number and size, and located randomly over acrosomal region; B3), tapered head (B4), pyriform head (B5), constricted or hour-glass head (B6), and degenerate head (B7). 

(C) Acrosomal defect in dorsoventral (C1 and C2) and side (C3) views. This morphologic defect is termed “knobbed acrosome.” 

(D) Proximal (D1) and distal (D2 and D3) cytoplasmic droplets. 

(E) Abnormalities of the midpiece, including segmental aplasia of the mitochondrial sheath (E1), roughed midpiece from uneven distribution of mitochondria (E2), enlarged mitochondrial sheath (E3), bent midpiece (E4–E6), and double midpiece/double head (E7). 

(F) Bent tail (or hairpin tail), involving the mid-region of the principal piece (F1–F4) or with a singular bend (F5) or proximal bend (F6) involving the midpiece-principal piece junction. 

(G) Coiled tail, with the tail tightly encircling the head (G1) or the tail coil not encircling the head (G2 and G3). 

(H) Fragmented sperm, including head detachment (termed detached heads or tailless heads; H1) or fragmentation at the level of the annulus (H2). Fragmentation can also occur at other sites, as shown in F4. 

(I) Premature germ cells with a single nucleus (I1) or multiple nuclei (I2).
tential, including the spatial and temporal changes, energetics, and gaseous environment involved, will we be able to reliably manipulate spermatozoa to meet the growing needs within the equine breeding industry. Goals might include devising methods for long-term cooled semen preservation, improving the fertility of cryopreserved semen, and incorporation of in vitro fertilization (both conventional in vitro fertilization and intracytoplasmic sperm injection) into commercial programs. Although these might seem to be lofty goals, a more absolute understanding of spermatozoal structure and function would certainly take a lot of the “guess work” out of current approaches to analysis and manipulation of equine spermatozoa.

Attempts to gain a better understanding of equine spermatozoa solely from extrapolation of data acquired from other mammalian species are likely destined to failure because of the well-known species differences in spermatozoal attributes and physiology. Nonetheless, much information derived from other species may have relevance to equids and should be investigated for applicability. Examples include identification of candidate genes for specific spermatozoal traits, targeted mutation of genes for specific proteins to study the resulting effect on reproductive function, and use of gene silencing agents for regulatable ablation of gene function. Incorporation of molecular techniques would seem to be the key to elucidation of mechanisms that control spermatozoal development and function in stallions.

Indeed, the “omics” era (e.g., genomics, toxicogenomics, transcriptomics, metabonomics, and proteomics) is on us. Technological progress in the field of molecular genetics has been remarkable and has opened the door to new paradigms for the study of spermatozoal function and dysfunction. Significant inroads have been made in horse genome mapping in recent years, including availability of synteny, linkage, radiation-hybrid, cytogenetic, and human-horse comparative maps. The National Institutes of Health recently reported (www.nih.gov/news/pr/feb2007/nhgri-07.htm) that the 2.7-billion DNA base-pair horse genome has been sequenced and partially assembled, and the first draft of the sequence is now accessible through public databases; hence, the importance of bioinformatics to biological research. Continued progress in genomic research will lead to isolation and identification of genes responsible for spermatozoal development and function, as well as genetic factors that underlie various types of reproductive failure. High-throughput microarray technology (cDNA and oligonucleotide) is being used with increased intensity to study differential gene expression. Reverse transcription and amplification steps are being applied to quantify gene activity in targeted cells or processes and to unravel the complexity of genes. The large-scale information obtained from these emergent technologies is being applied to male fertility, including that of horses. Interpreting of such expression profiling and its biologic significance, however, can be a quagmire, and continued effort is required to hurdle this obstacle. Given the intensity of study in this area, the basic mechanisms that control gene expression will emerge in the years ahead, and genetic markers for stallion fertility may one day abound. Robust mass spectrometry proteomics methodologies allow inventory of proteins in cells, organelles, and body fluids and offer the potential for identification of biomarkers for diagnostic tests.

As with humans and other animal species, the impacts that environmental chemicals have on stallion fertility deserve attention. An apparent decline in spermatozoal quality and quantity of men has been described in recent years. Increased rates of other reproductive tract problems, such as cryptorchidism and testicular cancer, have also been reported. Environmental reproductive toxicants are considered to be a major contributor to these patterns. Both cDNA microarray and real-time reverse transcriptase-polymerase chain reaction approaches have revealed differential expression profiles in some spermatogenesis-related genes (i.e., heat shock protein 70–2, insulin growth factor binding protein 3, and glutathione S transferase pi) after toxicant exposure, so genes such as these may serve as useful biomarkers when screening for testicular toxicity.

A better understanding of the molecular mechanisms regulating spermatozoal development and function will likely lead to new diagnostic techniques and therapeutic strategies for reduced fertility in stallions and may possibly translate to methodologies designed to curb gonadal aging. Such progress can only be curtailed by funding deficits or diverted interests of investigators in the years ahead. Undoubtedly, future generations will look beyond descriptive morphology and motility in characterizing structural and functional features and deficiencies of spermatozoa. In addition, they will likely use the spermatozoon as a vehicle for production of transgenic horses, as well as spermatogonial stem cell transplantation or testicular grafting techniques to study testicular function or to propagate a genetic line.

It seems befitting to conclude this manuscript with a quote from Professor Storey, as presented in the first reference entitled “Interactions between gametes leading to fertilization: the sperm’s eye view” . . . “the reactions that lead to fertilization of the mammalian egg by its conspecific sperm are many, complex, and exquisitely coordinated. One might add that they are also vital.”

Financial assistance was provided by the Abercrombie Foundation, the Patsy Link Endowment Fund, Texas A&M University; the Shining Spark-Carol Rose Stallion Reproduction Fund (through the American Quarter Horse Association); the Azoom, Corona Cartel, and Teller Cartel syndicates (through Lazy E Ranch); Black Rock Ranch; Cold-
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