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Sperm Conjugation in Mammal Reproductive Function: Different Names for the Same Phenomenon?

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SUMMARY

In many mammalian and non-mammalian species, mature sperm interact within the female reproductive tract or inside the epididymal lumen using cohesive forces. This phenomenon, known as ''sperm conjugation,'' is sometimes confused with sperm agglutination, which is the result of the interaction of epididymal or ejaculate spermatozoa upon release into culture medium. In addition to ''agglutination,'' the terms ''association,'' ''rouleaux,'' or ''rosettes'' are employed interchangeably to describe the conjugation phenomenon, which inevitably causes confusion due to the non-unifying nomenclature. This variety of descriptions is likely due to a poor understanding of the molecular mechanisms involved in such conspicuous cell-cell interaction as well as the different morphologies that result from such interactions among species. Here, we summarize the published data regarding mammalian sperm conjugation, considering the organisms in which sperm interaction was observed; the particular terminology employed; findings regarding the components that enable sperm to adhere; sperm behavior when deposited in the female reproductive tract; and hypotheses formulated to clarify the biological function and, when known, the mechanisms for sperm interaction. We also propose a new classification system for this phenomenon that might clearly unify the criteria used to describe this behavior.

Sperm conjugation is more widespread among mammals than previously thought.

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INTRODUCTION

Sperm conjugation occurs inside the female genital tract or during epididymal transit in many mammals. Such interaction of two or more live sperm usually occurs via their heads, and is prevalent in the distal segments of the epididymis and vas deferens or inside the female genital tract. The timing and location of this phenomenon implies that the behavior is part of the epididymal maturation process, yet little is reported about sperm conjugation, particularly as sperm typically disassociate into single cells before reaching the site of sperm storage or fertilization within the female body.

This review examines the current information related to the phenomenon by compiling the primary literature, describing the main types of sperm conjugation, and

summarizing the proposed biological purposes. Possible molecular mechanisms involved in these sperm arrangements are also mentioned, when known.

The coverage of mammalian sperm conjugation is scant in the scientific literature (e.g., databases such as PubMed). Higginson and Pitnick (2011) recently reviewed which sperm interactions lead to a cooperative role, defining ''sperm conjugation'' as an unusual sperm behavior involving the physical association of two or more spermatozoa for motility or transport through the female reproductive tract (Pitnick et al., 2009a). These authors classified conjugations as primary or secondary, depending on their origin: Primary sperm conjugations are derived from spermatogenic processes whereas secondary conjugations are the result of post-spermatogenic events. These phenomena are widely observed in many taxonomic groups (Pitnick et al., 2009b). Immler (2008) summarized different forms of sperm cooperation, and indicated which sperm form pairs among rodents and American marsupials. Higginson and Pitnick (2011) also provided a table summarizing examples of mammalian sperm conjugation, such as ''rouleaux,'' ''pairs,'' ''bundles,'' and ''trains.'' Despite the update, however, Higginson and Pitnick were not comprehensive, omitting or overlooking examples such as from flying squirrels (Martan and Hruban, 1970), primates (Phillips and Bedford, 1987), and rodents (Fornes and Burgos, 1990, 1994; Monclus et al., 2007; Monclus et al., 2010).

The main limitation when searching for references regarding sperm conjugation is a lack of unifying terminology that describes these phenomena. Terms such as ''pairs,'' ''couples,'' ''rouleaux,'' ''rosettes,'' ''bundles,'' and ''trains'' are often used to describe the arrangement of sperm —which depend on sperm morphology—rather than the association itself. Thus, in the absence of a uniform lexicon, the omission of references that report sperm conjugation within a species is inevitable; nevertheless, we apologize if we also omit any citations from this review. A second obstacle to inclusion in this review is the source of the article itself: we restricted our searches to full-text articles or abstracts that were written in English and were available through online sources.

EPIDIDYMAL MATURATION IS ESSENTIAL FOR SPERM CONJUGATION

Sperm conjugation is often observed in the more-distal epididymal regions or inside the female genital tract, so we infer that these cell populations have sufficiently matured during their passage through the epididymis. When sperm leave the testis, they are neither motile nor capable of fertilizing the egg; instead, sperm require post-testicular modifications that are essential for acquiring fertilizing capacity. The journey through an environment that provides adequate conditions to support those changes is key for their maturation, which include modifications to the lipid composition of the plasma membrane, an increase in the negative net charges and disulfide bonds, and elimination and remodeling of surface proteins (Sullivan et al., 2005).

Each region of the epididymis facilitates different processes that ultimately concentrate, mature, transport, and store the sperm (Robaire and Hermo, 1988; Cornwall et al., 2002). Differential gene expression along the epididymis supports the region-specific activities of this organ on the sperm (Jervis and Robaire, 2001; Johnston et al., 2005). Maturing epididymal sperm experience a steadily changing environment that is isolated from the blood via the blood-epididymis barrier, a structure that provides anatomical, physiological, and immunological limits to assure gamete protection before ejaculation as well as control the function and integrity of the epididymis (Mital et al., 2011).

Sperm progression from the caput to the cauda epididymis is a passive process because sperm have not yet developed motility; they instead rely on the flow of epididymal secretions. Only after their complete passage through the epididymis do mammalian sperm become motile and achieve the ability to undergo capacitation and to interact with the egg. This maturation process depends on interactions with epididymal fluid, specifically with its proteins, because sperm cells are transcriptionally inactive (Dacheux et al., 2005). These secreted proteins are either added to the sperm plasma membrane or help remodel the existing membrane proteins. Their expression by the epididymal epithelium is often under the control of androgens (Robaire and Viger, 1995).

Post-testicular sperm differentiation can last from several days to weeks, depending on the species (Yanagimachi, 1994; Toshimori, 2003); a summary of the principal findings on this topic was reviewed by Cornwall (2009). An amazing improvement in the knowledge of sperm biology has been obtained from proteomic, secretomic, and transcriptomic techniques (Guyonet et al., 2011; Baker et al., 2012; Dacheux et al., 2012), yet the molecular mechanisms involved in the process that transforms immature sperm into gametes capable of fertilization have not been completely elucidated. Associations between sperm are generally observed in the most-distal segments of the epididymal tract, in which the gametes are completely mature, so what is the relationship between epididymal sperm maturation and sperm conjugation?

SPERM CAPACITATION

Mammalian sperm are unable to fertilize an egg at the time of ejaculation. They acquire their full fertilizing capacity after a period in the female genital tract, during which many biochemical and physiological changes occur; these events are part of an overall process called capacitation, which was first described more than 60 years ago (Austin, 1951; Chang, 1951). Capacitation requires a combination of sequential and concurrent processes that mainly target the plasma membrane of the sperm, involving the loss of components from seminal plasma, modification of the lipid composition of the plasma membrane, increased permeability to calcium, increased intracellular pH, redistribution of surface components, increasing motility

(hyperactivation), elevation of the intracellular concentration of cyclic AMP (cAMP), and the induction of sperm protein tyrosine phosphorylation (de et al., 1997; Visconti and Kopf, 1998). Both the sperm head (preparation for the acrosomal reaction) and the flagellum (motility changes) are affected by capacitation. The time required inside the female genital tract to reach the capacitated status differs among species, ranging from 1 to 2 hr in mice to 6 to 7 hr in humans (Davis, 1981). Only sperm that have completed capacitation can undergo the acrosomal reaction when they contact the outer layers of the egg.

In species whose sperm undergo conjugation, individual sperm cannot fertilize an egg until the conjugates are disassembled. This implies that the sperm within a conjugate must also be able to separate to move alone. Conjugate disassembly generally occurs within the female reproductive tract, at the time and place of capacitation, although the stimulus that triggers sperm release from the conjugate is unknown.

MAMMALIAN SPERM CONJUGATION

Sperm conjugation will be discussed according to the nomenclature used in the literature for different species. Table 1 summarizes the main characteristics presented below.

Sperm Stacked in Rouleaux

Guinea Pig

The first reference to sperm conjugation in the available literature is a description of ''rouleaux'' in *Cavia porcellus*, (Simeone and Young, 1931)—although more recently, Cooper et al. (2000) reported that this phenomenon does not occur in all guinea pigs: sperm of the non-domesticated guinea pig from South America, *Cavia aperea*, agglutinate into rouleaux, whereas these configurations are not observed in *Galea musteloides*. Guinea pig rouleaux are formed by stacking sperm with spoon-shaped heads, organized with the convex surface of one head contacting the concave surface of another. Filamentous material was noted close to the plasma membrane adhesion zones, filling the space between sperm heads. The tails of the assembled sperm can move freely, favoring the displacement of these rouleaux in culture media (Fawcett and Hollenberg, 1963).

Sperm rouleaux first appear at epididymal segment four, suggesting that the attainment of a certain level of sperm maturation is required for association in the guinea pig (Fawcett and Hollenberg, 1963). *C. porcellus* rouleaux dissociate progressively with time both in vivo (uterine cavity) after ejaculation and also in vitro (culture media). Williamson et al. (1980) inseminated females with sperm associated in rouleaux from the vas deferens or with immature sperm (not associated), and reported lower fertility using single sperm. Tung et al. (1980) tested the dispersion of sperm rouleaux obtained from the caudal epididymis in the presence of auto-antibodies generated against guinea pig spermatozoa (Fig. 1F), and concluded that the sperm conjugation phenomenon prevents premature acrosome reaction and preserves sperm viability. WH-30, a guinea pig sperm surface protein, was identified as a participant that maintains the sperm rouleaux configuration (Flaherty et al., 1993).

Armadillo

There is only one reference of rouleaux formation in the naked-tail armadillo, *Cabassous unicinctus*. Heath et al. (1987) described the presence of rouleaux inside the epididymal duct, with sperm heads arranged in stacks of four to ten cells and their plasma membranes closely juxtaposed over the acrosomal region.

Loris

In the prosimian primate known as loris, *Nycticebus coucang*, sperm enter the epididymis as single cells and then stack in rouleaux (Fig. 1G). When the rouleaux reach the cauda epididymis, however, all the spermatozoa separate into single cells (Phillips and Bedford, 1987). No functional significance was proposed for this transient phenomenon.

Sperm Pairs

''Sperm pairing'' is commonly used to describe sperm conjugation in New World marsupials, but not Australian marsupials (Temple-Smith, 1987). Krause and Cutts (1979) observed sperm pairing in the caudal region of the *Didelphis virginiana* epididymis using a scanning electron microscope. These sperm pairs form with the adjacent plasma membranes over the acrosomal region in close apposition. The presence of paired sperm is first observed in the lower corpus region of the epididymis (Temple-Smith and Bedford, 1980), and the sperm pairs separate in the oviduct and during fertilization (Rodger and Bedford, 1982). Similar pairings were observed in the caudal epididymal content of the wooly opossum, *Caluromys philander* (Phillips, 1970), and the marsupial *Didelphis azarae* (Orsi et al., 1981). Bedford et al. (1984) proposed that sperm pairing provided an acrosome-protective function for marsupial sperm, specifically against the preoviductal environment of the female reproductive tract.

Sperm pairing also occurs in *Monodelphis domestica* (Fig. 1H), beginning in the proximal corpus of the distal epididymis and reaching a maximum of 80% in the caudal storage region. Sperm pairs are present in the ejaculate, and then they separate at the oviduct ampulla. Motility is improved when *M. domestica* sperm are paired, even in viscous medium, compared to isolated cells (Moore and Taggart, 1995).

Sperm Twisted in Bundles

A particular type of sperm conjugation has been reported in the echidna, *Tachyglossus aculeatus*. Djakiew and Jones (1981, 1983) described these conjugates found in the terminal segment of the echidna epididymis as ''sperm

TABLE 1. Summary and Proposed Function of the Secondary Sperm Conjugation Found Inside the Epididymis, Ejaculate, or Female Genital Tract

Species	Type of conjugation	Isolated from/characteristics	Proposed function	Other characteristics
Guinea pig (*Cavia porcellus*)	Rouleaux	Distal epididymis/female genital tract/in vitro culture media	Acrosome protection/preserve sperm viability in the female tract	Androgen dependence/ WH30 protein
Non-domesticated guinea pig (*Cavia aperea*)	Rouleaux	Distal caput	Preserve acrosome integrity	
Armadillo (*Cabassous unicinctus*)	Rouleaux	Vas deferens		
Loris: primate (*Nycticebus coucang*)	Rouleaux	Appears in the caput epididymis/ disappears before reaching the cauda		
Opossum: Marsupial (*Monodelphis domestica*)	Pair (head-to-head)	Distal epididymis/ejaculate	Acrosomal protection/increase the displacement velocity	Separates in oviduct
Opossum: Marsupial (*Didelphis virginiana*)	Pair (head-to-head)	Distal epididymis	Acrosomal protection/maturation	
Opossum: Marsupial (*Didelphis azarae*)	Pair (head-to-head)	Distal epididymis		
Echidna: Monotreme (*Tachyglossus aculeatus*)	Bundles (up to 100 sperm)	Epididymis terminal segment/ejaculate	Sperm competition/prevent premature capacitation/ sperm storage	Two epididymal proteins (60 and 76 kDa) are involved
Mouse: Rodent (*Mus musculus*)	Sperm groups/rosettes (10–12 sperm)	Distal epididymis/Vas deferens/ejaculate	Probably increase the displacement velocity/acrosome protection	
Rat: Rodent (*Rattus norvegicus*)	Sperm groups/rosettes (10–12 sperm)	Distal epididymis/Vas deferens/ejaculate	Increase the displacement velocity/acrosome protection	Epididymal serpins is involved
Wood mouse: Rodent (*Apodemus sylvaticus*)	Trains (100–1000 sperm)	In vitro culture medium/ejaculate/ female genital tract	Increase the displacement velocity/competition	
Deer mouse: Rodent (*Peromyscus maniculatus/polionotus*)	Sperm aggregations (2–40 sperm)	In vitro culture medium/ejaculate	Competition	
Flying squirrel: Rodent (*Glaucomys volans*)	Cylindrical bodies/rouleaux	Caput/corpus and cauda, respectively		
Fox squirrel: Rodent (*Sciurus niger*)	Cylindrical bodies/rouleaux	Caput/corpus and cauda, respectively	Maturation	
Eastern grey squirrel: Rodent (*Sciurus carolinensis*)	Cylindrical bodies/rouleaux	Caput/corpus and cauda, respectively	Maturation	
Chinchilla: Rodent (*Chinchilla sp.*)	Cylindrical bodies	Appears in the middle caput/disappears in the lower caput and corpus	Maturation	

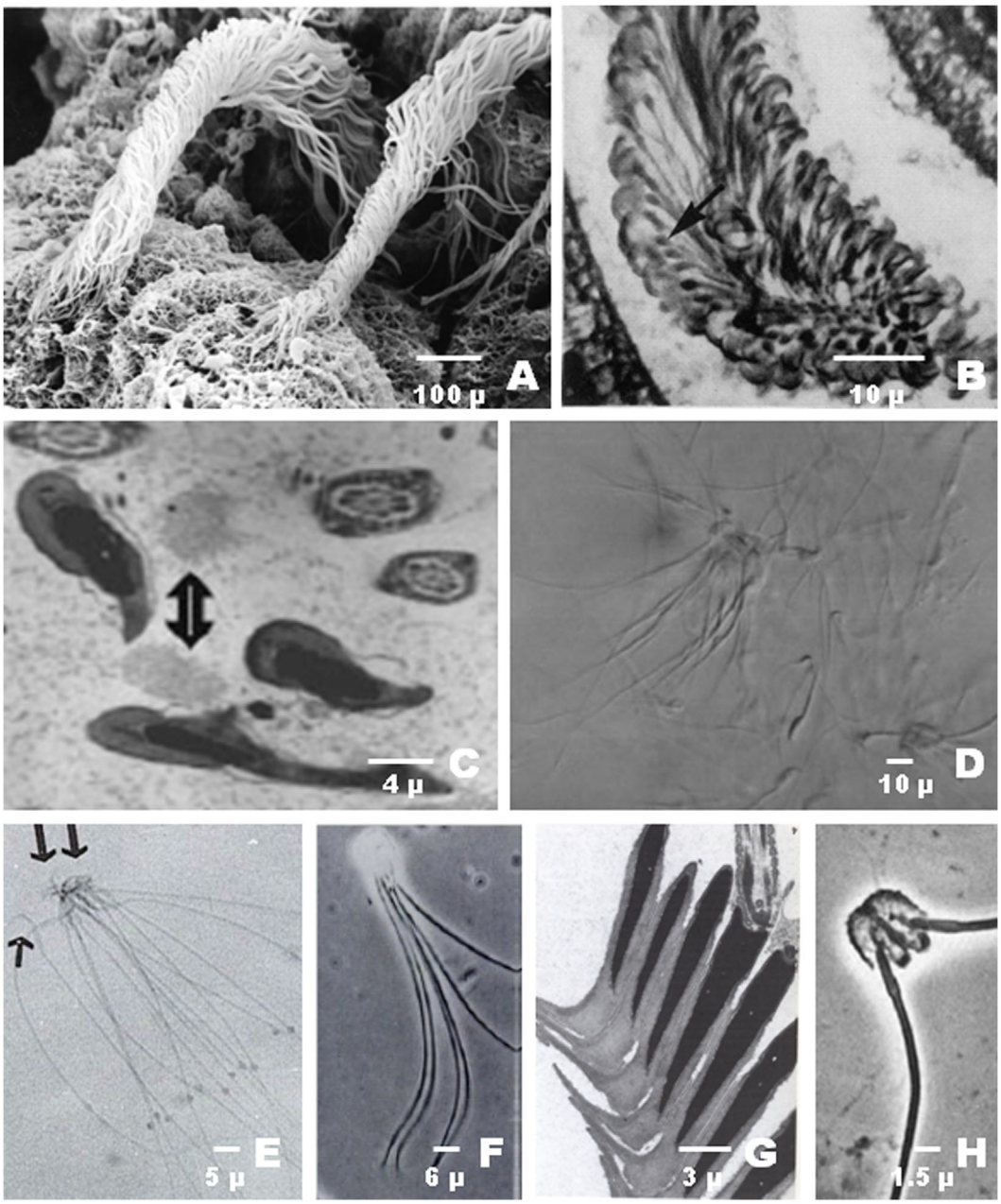

Figure 1. Mammalian sperm conjugation. **A**: Scanning electron micrograph of bundles of echidna spermatozoa in the terminal segment of epididymal duct (×2,900) (Courtesy of Dr. Russell Jones). **B**: Longitudinal section through the cylindrical body in the epididymal lumen of Sciurus squirrels (×680) (Martan et al., 1970). **C**: Transmission electron micrograph of fixed mouse sperm rosettes. An electron-dense material between cells was detected in samples obtained from the caudal epididymis (×8,000) (Monclus et al., 2007). **D**: Mouse sperm rosette obtained by puncturing the caudal epididymis (×400) (Monclus et al., 2007). **E**: Rat sperm rosette showing a positive reaction agglutinating material at the head-to-head region (double arrows), but no reaction in an isolated sperm head (single arrow) (×450) (Fornes and Burgos, 1990). **F**: Phase-contrast micrograph of a guinea pig sperm rouleaux (×1,500) (Tung et al., 1980). **G**: Transmission electron micrograph of loris (*Nycticebus coucang*) spermatozoa obtained from the caput epididymis, showing the heads associated by a junction complex between plasma membranes (×21,000) (Phillips and Bedford, 1987). **H**: Sperm pair in *Monodelphis domestica*, an American marsupial (×580) (Moore and Taggart, 1995). Scale bars indicate estimated dimensions. All images reproduced with permission from their specific sources.

bundles.'' Each bundle consists of more than 100 spermatozoa wrapped in a helical fashion (Fig. 1A), bound together by an electron-dense material. Sperm motility was retained after dilution of the luminal content in a Krebs-Ringer phosphate solution. Individual sperm from the initial segment of the epididymis were found to be slower than sperm bundles obtained from the terminal segment, and bundles obtained from the distal epididymis persisted for

at least 2 hr during in vitro incubation in capacitating media (Jones et al., 2007).

Johnston et al. (2007) proposed many possible explanations for *T. aculeatus* sperm bundle formation, based on video-microscopy analysis of sperm bundle movement. These hypotheses included sperm competition and the reduction or avoidance of premature capacitation, as well as a novel strategy for sperm storage in the female genital tract. Nixon et al. (2011) supported the hypothesis that bundle formation is a sperm-cooperative behavior in echidna. Considering that bundle dispersion in the female genital tract is a time-dependent process, Jones et al. (2009) further proposed that separation from the bundle represents an early phase of capacitation that may facilitate the unmasking of zona pellucida ligands on the sperm head, leading to proper fertilization.

Sperm Conjugation in Rodents

Muridae

Sperm conjugating into pairs, bundles, or rouleaux is generally accepted in the literature for many mammals—except within the Rodentia order, suborder Myomorpha; specifically in the Muridae family. The first studies reporting sperm conjugation were conducted by Fornes and Burgos (1990, 1994): These authors obtained samples of epididymal content by puncturing the epididymis of the rat, *Rattus norvegicus*, at different regions, and placed each onto a small drop of balanced salt solution (Radigue et al., 1988). Samples from the caput and corpus showed individual sperm with a circular movement (caput) or circular and progressive motility (proximal corpus). Samples obtained from the distal corpus or cauda, however, revealed sperm conjugations formed by many motile sperm joined along their heads, forming what Fornes and Burgos (1990) described as a ''rosette.'' Rosettes consist of a group of approximately a dozen sperm joined by their heads and with their tails free (Fig. 1E). Cysteine-rich secretory protein 1 (CRISP1) was later found to be present in the electron-dense material that joins the sperm heads (Fornes and Burgos, 1994).

Many years later, our group described the existence of a similar phenomenon in the mouse, *Mus musculus* (Monclus et al., 2007); we also referred to this sperm conjugation as ''rosettes'', and detailed their morphology using different microscopy techniques (Fig. 1C and D). Many similarities between mouse and rat rosettes were observed, particularly in their morphology and their presence at the distal regions of the epididymal lumen. While analyzing the mechanism of rosette assembly in vitro (see below) using sperm from the Wistar rat (*R. norvegicus*), we could not determine if rosettes move faster than isolated sperm (Monclus et al., 2010); therefore, we could not establish if sperm conjugation provided an advantage in sperm competition. We instead proposed that sperm rosettes play a protective role that prevents a premature acrosome reaction during epididymal sperm storage (Monclus et al., 2010).

Moore et al., (2002) described exceptional sperm cooperation in the wood mouse, *Apodemus sylvaticus*. When sperm obtained from the cauda epididymis were released into fertilization media, they move as single cells. Within 1 to 5 min, however, sperm cells modify their heads by ''opening'' their apical hook. This physical modification allows sperm to link into motile clumps of 10–50 cells, which progressively increased to hundreds and thousands of cells. The authors called these structures ''sperm trains,'' and noted that these structures moved faster than single cells, even in a viscous medium. Dispersion of a sperm train began after 30 min, and was complete in approximately 90 min. Those sperm removed from the train underwent a premature acrosome reaction, compromising their own fertility; on the other hand, their time associated with the train helped other sperm reach the egg. This phenomenon represents cooperation and altruistic behavior at the cellular level.

A similar behavior was observed in deer mice, *Peromyscus polionotus* and *Peromyscus maniculatus* (Fisher and Hoekstra, 2010): Highly motile sperm were obtained from the cauda epididymis or from ejaculate. Within a few minutes in an adequate medium, these cells began to form motile aggregates of 2 to 40 sperm. The aggregates began to disperse after 40 min, and the dispersion was complete in 3 hr. The authors proposed a cooperative behavior that evolved in *P. maniculatus* in response to male competition because sperm from the same individual readily aggregates, whereas the speed of displacement within a potential hostile environment is favored in the monogamous *P. Polionotus*.

Train and aggregate formation (in the wood mouse and deer mouse, respectively), seem to be completely different from rosettes (house mouse and rat). The main difference lies in when sperm conjugation occurs: rosettes are present inside the lumen of the most distal epididymal regions and disperse upon release from the epididymis, whereas trains and aggregates are produced only when sperm are released from the epididymis—which presumably occurs inside the female tract. The observations made in relation to the speed of displacement and dispersion of sperm from these post-epididymal conjugations suggests a reproductive advantage based on cooperation and altruism.

Firman and Simmons (2009), however, stated that sperm conjugation is not a common characteristic in house mouse ejaculates. Indeed, the sandy inland mouse from Australia (*Pseudomys hermannsburgensis*) exhibited no evidence of motile sperm conjugation in vivo or in vitro (Firman et al., 2013). They concluded that these sperm, with a characteristic three apical hooks, do not favor the formation of motile conjugates.

Squirrels

In 1970, Martan and Hruban described the presence of cylindrical structures inside the epididymal caput of the flying squirrel, *Glaucomys volans*. These ''cylindrical bodies'' were formed by sperm whose tails adhered in the centre of the cylinder, with heads oriented toward the epididymal epithelium. As they progress along the

epididymis, the cylinders fragment into individual sperm or sperm clustered in rouleaux. A similar phenomenon was reported in the fox squirrel, *Sciurus niger*, and Eastern gray squirrel, *Sciurus carolinensis* (Martan et al., 1970). The authors suggested that epididymal sperm maturation includes modifications in sperm membrane properties that promote adhesion (Fig. 1B), and related the formation of cylindrical bodies to epididymal maturation because they observed a displacement of the cytoplasmic droplet as sperm journeyed through the organ.

Chinchilla

Martan also described cylindrical structures in the middle of the caput epididymis in the genus Chinchilla (Martan, 1970). The sperm adhere by their tails, with their heads mostly oriented in the same direction. The cohesive properties of epididymal sperm decrease as they mature, and the cylindrical structures gradually dissociate. Disorganized masses of spermatozoa with randomly orientated heads are found in the corpus epididymis.

HUMAN SPERM?

Among the mammals, the question of sperm conjugation in humans is of particular interested; unfortunately, the scenario is more complex. First, obtaining samples from the epididymis proves ethically challenging, so few studies are available. Bedford et al. (1973), however, analyzed several parameters of sperm cells obtained at different levels of the epididymis, and reported no evidence of sperm interaction.

Ex vivo, human sperm are reported to form disordered clumps—a phenomenon termed ''agglutination'', which is observed in many mammals from fresh semen as well as inside the female genital tract. This process reflects the tendency of sperm to interact with each other in response to a change in their environment. The *World Health Organization Laboratory Manual for the Examination and Processing of Human Semen*, Fifth Edition (2010) states that ''agglutination specifically refers to motile spermatozoa sticking to each other, head-to-head, tail-to-tail, or in a mixed way.'' This manual also recommends that agglutination grades for ejaculated human sperm be recorded according to Rose et al. (1976)—as an indicator of abnormality that may affect sperm function. For example, agglutination was first flagged as an indicator of immunologic infertility due to the presence of antibodies against sperm, which affects many aspects of the sperm function (movement, binding, fusion, and ovum penetration) required for fertilization (reviewed in Restrepo and Cardona-Maya, 2012).

In contrast to the relationship between sperm conjugation and reproductive advantage regarding sexual competition among males, competition among human sperm is centered predominantly in psychology, physiology, anatomy, and behavior rather than specific sperm interactions (Shackelford and Goetz, 2007; Goetz et al., 2008). Therefore, we assume that agglutination of human sperm is more likely linked to pathology than an improvement in male reproductive performance.

SPERM AGGLUTINATION AFTER EPIDIDYMAL CONTENT DILUTION

Sperm agglutination is well documented by livestock breeders and researchers as a phenomenon that occurs after diluting semen or epididymal sperm in a defined medium. Surprisingly, a particular and specific culture medium causes agglutination of sperm for individual species, which is summarized below.

Boar

Dacheux et al. (1983) described the occurrence of sperm agglutination when epididymal content is diluted and incubated in Krebs-Ringer bicarbonate medium at 37°C, with a maximal agglutination after 1 hr of incubation. This agglutination consists of groups of 2 or 3 sperm joined at their heads. Briz et al. (1995) reported three agglutination patterns in the boar: head-to-head, tail-to-tail, and head-to-tail. The percentage of each pattern of agglutination depended on the epididymal region where sperm were collected.

Dacheux et al. (1983) also determined that sperm agglutination was prevented or avoided when caudal epididymal proteins were added before or during the sperm incubation. These results suggest the existence of an epididymal protein with anti-agglutinin properties in cauda and ejaculate. Harayama et al. (1994) later identified such an anti-agglutinin boar sperm protein of 25 kDa that was also capable of maintaining sperm progressive motility in vitro.

Bull

Senger and Saacke (1976) observed that incubating bull sperm in medium containing bovine serum causes head-to-head sperm agglutination—an orientation that maintains acrosome integrity. Aalseth et al. (1978) obtained similar results using a semen extender containing egg yolk. In both cases, agglutination reversed after 9 hr.

Verberckmoes et al. (2004) tested if small amounts of blood from the artificial insemination procedure may affect the fertilizing capacity of inseminating sperm by adding blood to Hepes-TAPL medium. Indeed, the presence of blood increased the head-to-head sperm agglutination, whereas agglutination was not observed in the absence of additional blood or serum. Therefore, such agglutination is not harmful; indeed, it may be protective since the acrosome membrane remained intact. More recently, Yang et al. (2012) observed a similar phenomenon using a sperm diluent containing egg yolk-citrate and incubating at 22°C.

Ram

Dott and Walton (1960) observed the formation of clumps of motile sperm after diluting and washing ejaculated ram

sperm. Several years later, Roy et al. (2014) described the auto-agglutination of cauda epididymal sperm incubated in the presence of sugars and other divalent cations—specifically copper—in the culture medium. This Cu^{2+}-dependent cellular agglutination was mediated by the interaction of a cell-surface lectin with its ligand on the surface of the neighboring sperm.

Stallion

Pickett et al. (1975) examined the effect of sperm extender composition on staillion sperm agglutination. Veeramachaneni et al. (2006) later proposed that losses of seminiferous tubule integrity can cause immune disorders or granulomatous reactions that produce pro-agglutination anti-sperm antibodies in semen for the rest of the stallion's life.

Hamster

Sperm obtained from the caudal epididymis of hamsters (the golden hamster, *Mesocricetus auratus*, or the Chinese hamster, *Cricetulus griseus*) also undergo agglutination along their heads when incubated in vitro under capacitating conditions. When motility becomes hyperactivated, however, the sperm clumps breaks apart (Yanagimachi, 1982; Yanagimachi et al., 1983; Suarez, 1988). Whether or not this behavior is observed in vivo during capacitation inside the female genital tract is not clear.

PROPOSED MECHANISM OF SPERM INTERACTION

Sperm conjugation—be it ordered or random—has been observed in different mammals, yet little is known about how spermatozoa interact with each other. Proposed mechanisms within the literature appear to be as diverse as the terminology used to describe the phenomenon.

In echidna, sperm pass through the isthmus (narrow zone of the epididymis) as individual cells. When they reach the terminal segment of the epididymis, however, the sperm heads adhere along their rostral ends. A mass of proteins maintains this interaction, resulting in a spherical arrangement that facilitates the congregation of tails, forming a V-shaped bundle (Fig. 1A). Two proteins with molecular masses of 60 and 76 kDa are secreted by the epididymis in the regions where sperm join into bundles, suggesting that they may be involved in this phenomenon (Jones et al., 2007).

In the European wood mouse, cauda epididymal sperm released into fertilization media deploy their apical hook, thus favoring the physical connection to another deployed hook or tail. Actin filaments appear to be involved in this falciform head remodeling, which results in motile sperm trains of 100 to 1,000 cells. This phenomenon was also observed after mating inside the female genital tract (Moore et al., 2002).

An in vitro sperm re-association assay was performed to study the mechanism of sperm rosette arrangement in the rat (Monclus et al., 2010). The most motile fraction of individual caudal epididymis sperm, based on swim-up selection, were incubated with several protein fractions obtained from the epididymal cauda fluid. The fractions that promoted in vitro sperm re-association into rosettes contained proteins with molecular weights ranging from 40 to 80 kDa; two of the identified proteins, alpha-1 antitrypsin and Serpin 1F, a protein with an alpha-1 antitrypsin-like domain, are members of the serine protease inhibitor family. The Serpin 1F gene is expressed in the epididymis, prostate, and seminal vesicles, but not in the testis (Monclus et al., 2010), and was sensitive to flutamide administration (unpublished data). These serpins were hypothesized to participate in the assembly/disassembly of rosettes by modulating luminal protease activity (Cesari et al., 2010; Monclus et al., 2010). The proposed model suggested that epididymal sperm are stored in rosettes, with heads joined by an ''adhesive material.'' These clusters remain immobile due to factors present in the epididymal lumen (Usselman and Cone, 1983). Luminal proteases that can digest this adhesive material are blocked by a high concentration of inhibitors (e.g., serpins). Dilution of the epididymal fluid—at the time of ejaculation or by in vitro solutions—disrupts the stoichiometric balance between protease and inhibitor, allowing for the degradation of the adhesion material. Sperm motility is thus activated, and sperm separate themselves from the rosette in order to swim freely in culture medium or inside the female genital tract (Fig. 2).

BIOLOGICAL MEANING

The goal of sperm is to fertilize an egg. So what is the purpose of their temporary conjugation? For those sperm that undergo conjugation, could this process be a test that must be passed en route to their complete maturation?

In 2011, Higginson and Pitnick presented possible functions for sperm conjugation in a wide range of organisms. Included among these models are: *motility*—sperm conjugates possess a higher displacement speed than the isolated sperm; *competition*—increased chances to fertilize an egg; *protection* from possible spermicidal environments; *molecular exchange* between sperm that constitute the conjugate; and *facilitated egg penetration*. Many of these hypotheses have been accepted or rejected in different species, yet questions still remain. Some of these questions are rooted in the poor terminology used to describe this phenomenon, as first noted by Higginson and Pitnick (2011). The functions we noted for spermatic conjugation are summarized in Table 1.

DISCUSSION

This review had two main goals: First, the compilation of observations of mammalian sperm conjugation—which was challenging due to the lack of uniform terminology within the field. We cited numerous studies that were not considered in previous reviews on the subject, including

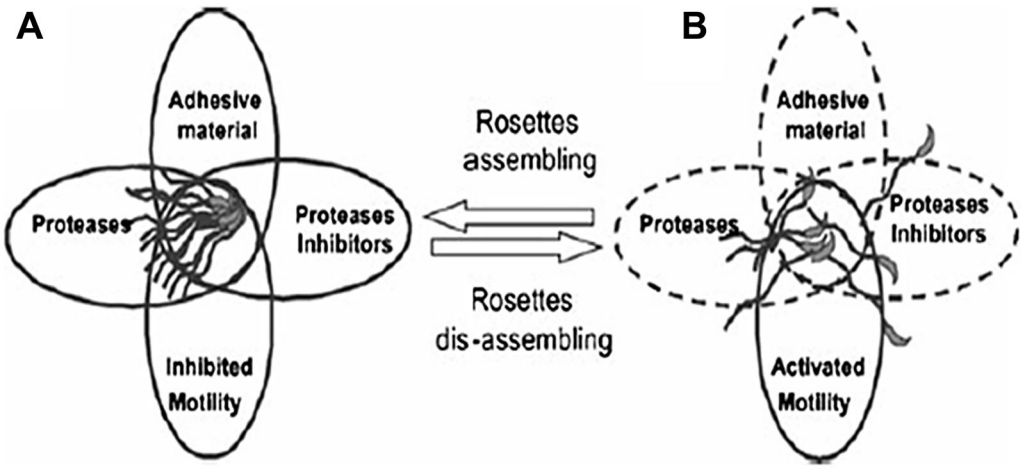

Figure 2. Proposed model explaining rat sperm conjugation into rosettes. **A**: Sperm are immotile inside the epididymal caudal lumen. Luminal proteases are inhibited by their endogenous antagonists (e.g., serpins), allowing the adhesive material between the sperm heads to maintain their association in a typical rosette morphology. **B**: Dilution of the epididymal caudal fluid in the female genital fluid or in chemically defined media leads to rosette disassembly. This process likely occurs as a result of protease activation and the degradation of the adhesive material that bridges the sperm heads and impedes motility (Monclus et al., 2010).

examples such as squirrels (Martan and Hruban, 1970; Martan et al., 1970), chinchilla (Martan, 1970), the loris (Phillips and Bedford, 1987), armadillo (Heath et al., 1987), and some species of cavia (Cooper et al., 2000); again, we apologize for omitting other published reports of this phenomenon. These observations led us to conclude that sperm conjugation is more widespread among mammals than previously thought. Second, and perhaps more difficult, is identifying the biological role for sperm conjugates among the different species. A question that arises is: Why must sperm be associated at the end of the epididymis or inside the female genital tract, only to disassociate before fertilization? The obvious answer is a selective advantage that allows the sperm to efficiently fulfill their mission of fertilizing an egg.

The morphology of the sperm conjugate is generally influenced by the shape of the sperm—specifically the head. Sperm heads of guinea pigs are morphologically similar to a spoon, thus the most effective interactions occur along the concavo-convex face, forming a rouleaux (Fawcett and Hollenberg, 1963). Most murine rodent sperm have falciform heads; indeed, sperm of the European wood mouse, Norway rat, and house mouse contact each other via the ventral region of their apical hook, which seems to be the most suitable site for reversible interactions to occur. In mouse and rat rosettes, the lateral faces are joined by material is present between sperm heads. In American marsupials, the close apposition of parallel acrosomal faces allows for sperm pairing via a slight head rotation, which precisely aligns the acrosomal surfaces (Bedford et al., 1984).

No consensus exists among researchers regarding the relationship between sperm conjugates and sperm competition. One reproductive advantage of sperm conjugation in a competitive mating system is the higher speed obtained by the conjugate versus an individual gamete. The increase in the displacement speed may be due to the greater thrusting force of the conjugated sperm, particularly in a viscous medium (Moore and Taggart, 1995; Moore et al., 2002). This model is supported in the echidna (Djakiew and Jones, 1983; Jones et al., 2007), American marsupials (Taggart et al., 1993), wood mouse (Moore et al., 2002), deer mouse (Fisher and Hoekstra, 2010), and rat, but not the house mouse (Immler et al., 2007). A controversial and counter example comes from non-domesticated guinea pigs: *C. aperea* exhibits a polygynous mating system—which implies a lower risk of sperm competition between different males—yet sperm agglutinate into rouleaux inside the epididymis; *G*. musteloides, on the other hand, has a promiscuous mating behavior with a higher competition risk, yet agglutination into rouleaux does not occur (Cooper et al., 2000).

Numerous studies have analyzed the relationship among the physical characteristics of the apical hook on a falciform rodent sperm, the tendency to conjugated sperm, and the risk of sperm competition. Immler et al. (2007) related sperm head morphometry to competition and cooperation, concluding that the shape and curvature of the apical hook of rodent sperm heads is influenced by the risk of sperm competition. On the other hand, a recent attempt to relate sperm competition (analyzed by relative testes mass), sperm quantity, and traits that determine ejaculate quality among 18 rodent species revealed that sperm competition favors an increase in both sperm numbers as well as the proportion of normal, motile and acrosome-intact sperm (Gómez et al., 2011). Varea-Sánchez et al. (2014) and Sandera et al. (2013) later proposed that sperm competition favors the production of more uniform sperm heads and flagella, which lead to enhanced swimming velocity—particularly in Apodemus

and Mus sperm. Unfortunately, these reports made no mention or consideration of the contribution of sperm conjugation in the parameters analyzed.

Another role proposed for sperm conjugation is the preservation of sperm integrity, for example, to hinder a premature acrosome reaction, which would result in the loss of fertilizing capacity. Spermatozoa represent an exceptional case in which a cell fulfils its function in an unfamiliar and hostile environment (Birkhead et al., 1993). Sperm conjugation as protection from the female reproductive tract fluid has been suggested for the opossum (Bedford et al., 1984), loris (Phillips and Bedford, 1987), rodents (Fornes and Burgos, 1994; Immler et al., 2007), and guinea pigs (Yanagimachi and Mahi, 1976; Tung et al., 1980; Cooper et al., 2000); additional protection from phagocytosis by polymorphonuclear leukocytes residing in the female genital tract was postulated for guinea pigs (Martan and Shepherd, 1973).

How sperm associate into a conjugate remains unclear, but reversal of this process is required for proper fertilization, thus minimizing polyspermy. Separation of sperm from the conjugate may lead to the damage of some sperm—up to 50% can be affected, resulting in premature acrosome reactions, as described in the wood mouse (Moore et al., 2002), as well as a loss in motility, as documented for one of the paired marsupial sperm (Rodger and Bedford, 1982; Moore and Moore, 2002). Such behavior is considered an example of gamete altruism since some of the sperm will benefit from the loss of others (Pizzari and Foster, 2008; Pizzari and Parker, 2009). One mechanism that facilitates the disassembly of epididymal rat rosettes involves the degradation of material between the sperm heads (Monclus et al., 2010). On the other hand, how conjugate dissolution occurs deeper within the female genital tract remains a mystery.

Sperm clearly exhibit a tendency to adhere to each other, as supported by their auto-agglutination behavior in culture medium. This behavior may be a consequence of their surface profiles, which likely contain molecules (lectins) whose function is enhanced by components in the media, leading to a higher affinity for surface molecules on neighboring sperm (Roy et al., 2014), or is a result of the dilution of epididymal factors with anti-sticking properties (Roy and Majumder, 1989; Harayama et al., 1994).

Recently, Babcock et al. (2014), studying the episodic rolling movement in mouse caudal sperm observed the occurrence of sperm to sperm attachment in culture media. They suggest the possibility that these transient sperm attachment prevents or avoid the sperm interaction to the oviductal reservoir favoring the progression through the female reproductive tract in vivo.

CONCLUSIONS

The classic definition of sperm conjugation is ''unusual sperm behavior where two or more spermatozoa physically unite for motility or transport through the female genital tract'' (Pitnick et al., 2009a). After reviewing all of the accessible material, we suggest expanding this to include sperm interactions formed within the epididymal lumen, in vitro after sperm release, or in the female genital tract. We also propose classifying the post-spermatogenic sperm conjugates according to the place and time at which conjugation occurs. We suggest using the term ''epididymal conjugation'' when an intimate association between sperm occurs inside the lumen of the epididymal tract. In most of the aforementioned cases, this conjugation occurs in the distal segments of the epididymis, which reinforces the relationship between location and maturation status. On the other hand, the term ''post-ejaculate conjugation'' should be used when sperm conjugation occurs within the female genital tract or is induced by incubating isolated epididymal sperm in a suitable culture medium.

Classifying based on the location of conjugate formation allows us to infer the various biological roles of sperm conjugation. The main function of conjugation in the epididymis, for example, is to protect sperm integrity during storage, thereby favoring their maturation. If the phenomenon occurs in the female genital tract, however, the functions may include an increase in rate of displacement in relation to non-conjugated sperm, protection against the aggression of the female genital tract, or even preparation for capacitation.

Further work is needed to clarify the evolutionary advantages of sperm conjugation, as well as to achieve a consensus regarding the terminology used to describe this phenomenon. We suggest using the term ''sperm conjugation,'' preceded by ''epididymal,'' or ''post-ejaculated'' to properly describe this phenomenon under all possible biological conditions.

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DEDICATION

In memory of Dr. Mario Hector Burgos, who recently passed away. He was a man with strong convictions and initiative and a pioneer in electron microscopy in Argentina. He founded the Institute of Histology and Embryology in 1957, where we are still working following his legacy.

REFERENCES

Aalseth EP, Senger PL, Becker WC. 1978. The relationship of sperm viability and concentration to serum-induced head-to-head agglutination of bovine spermatozoa. J Reprod Fertil 53:193–196.

Austin CR. 1951. Observations on the penetration of the sperm in the mammalian egg. Aust J Sci Res 4:581–596.

Babcock DF, Wandernoth PM, Wennemuth G. 2014. Episodic rolling and transient attachments create diversity in sperm swimming behavior. BMC Biol 12:67. doi: 10.1186/s12915-014-0067-3.

Baker MA, Nixon B, Naumovski N, Aitken RJ. 2012. Proteomic insights into the maturation and capacitation of mammalian spermatozoa. Syst Biol Reprod Med 58:211–217.

Bedford JM, Rodger JC, Breed WG. 1984. Why so many mammalian spermatozoa-a clue from marsupials? Proc R Soc Lond B Biol Sci 221:221–233.

Bedford JM, Calvin H, Cooper GW. 1973. The maturation of spermatozoa in the human epididymis. J Reprod Fertil Suppl 18:199–203.

Birkhead TR, Møller AP, Sutherland WJ. 1993. Why do females make it so difficult for males to fertilize their eggs? J Theor Biol 161:51–56.

Briz MD, Bonet S, Pinart B, Egozcue J, Camps R. 1995. Comparative study of boar sperm coming from the caput, corpus, and cauda regions of the epididymis. J Androl 16:175–188.

Cesari A, Monclus MA, Tejón GP, Clementi MA, Fornes MW. 2010. Regulated serine proteinase lytic system on mammalian sperm surface: There must be a role. Theriogenology 74:699–711.

Chang MC. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:697–698.

Cooper TG, Weydert S, Yeung CH, Künzl C, Sachser N. 2000. Maturation of epididymal spermatozoa in the nondomesticated Guinea pigs Cavia aperea and Galea musteloides. J Androl 21:154–163.

Cornwall GA. 2009. New insights into epididymal biology and function. Hum Reprod Update 15:213–227.

Cornwall GA, Lareyre J, Matusik P, Hinton BT, Orgebin-Crist MC. 2002. Gene expression and epididymal function. In: Robaire B, Hinton BT, editors. The epididymis: From molecules to clinical practice. New York: Kluwer Academic Press/Plenum Press Publishers. pp 169–199.

Dacheux JL, Paquignon M, Combarnous Y. 1983. Head-to-head agglutination of ram and boar epididymal spermatozoa and evidence for an epididymal antagglutinin. J Reprod Fertil 67:181–189.

Dacheux JL, Castella S, Gatti JL, Dacheux F. 2005. Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology 63:319–341.

Dacheux JL, Belleannée C, Guyonnet B, Labas V, Teixeira-Gomez AP, Ecroyd H, Druart X, Gatti JL, Dacheux F. 2012. The contribution of proteomics to understanding epididymal maturation of mammalian spermatozoa. Syst Biol Reprod Med 58:197–210.

Davis BK. 1981. Timing of fertilization in mammals: Sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proc Natl Acad Sci USA 78:7560–7564.

de Lamirande E, Leclerc P, Gagnon C. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 3:175–194.

Djakiew D, Jones RC. 1983. Sperm maturation, fluid transport, and secretion and absorption of protein in the epididymis of the echidna, Tachyglossus aculeatus. J Reprod Fertil 68:445–456.

Djakiew D, Jones RC. 1981. Structural differentiation of the male genital ducts of the echidna (Tachyglossus aculeatus). J Anat 132:187–202.

Dott HM, Walton A. 1960. Effects of dilution and washing on ram spermatozoa studied by the flow dialysis technique. J Reprod Fertil 1:350–367.

Fawcett DW, Hollenberg RD. 1963. Changes in the acrosome of the guinea pig spermatozoa during passage through the epididymis. Z Zellforsch Mikrosk Anat 60:276–292.

Firman RC, Simmons LW. 2009. Sperm competition and the evolution of the sperm hook in house mice. J Evol Biol 22:2505–2511.

Firman RC, Bentley B, Bowman F, Marchant FG, Parthenay J, Sawyer J, Stewart T, O'Shea JE. 2013. No evidence of sperm conjugate formation in an Australian mouse bearing sperm with three hooks. Ecol Evol 3:1856–1863.

Fisher HS, Hoekstra HE. 2010. Competition drives cooperation among closely related sperm of deer mice. Nature 463:801–803.

Flaherty SP, Swann NJ, Primakoff P, Myles DG. 1993. A role for the WH-30 protein in sperm-sperm adhesion during rouleaux formation in the guinea pig. Dev Biol 156:243–252.

Fornes MW, Burgos MH. 1990. Sperm association in the rat epididymis. Microsc Electrón Biol Celular 14:115–129.

Fornes MW, Burgos MH. 1994. Epididymal glycoprotein involved in rat sperm association. Mol Reprod Dev 38:43–47.

Goetz AT, Shackelford TK, Platek SM, Starratt VG, Mckibbin WF. 2008. Sperm competition in humans: Implications for male sexual psychology, physiology, anatomy, and behavior. Ann Rev Sex Res 18:1–22.

Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C, Luque-Larena JJ, Gomendio M, Roldan ER. 2011. Sperm competition, sperm numbers and sperm quality in muroid rodents. PLoS ONE 6:e18173.

Guyonet B, Dacheux F, Dacheux JL, Gatti JL. 2011. The epididymal transcriptome and proteome provide some insights into new epididymal regulations. J Androl 32:651–664.

Harayama H, Miyano T, Miyake M, Kusunoki H, Kato S. 1994. Identification of anti-agglutinin for spermatozoa in epididymal boar plasma. Mol Reprod Dev 37:436–445.

Heath E, Schaeffer N, Meritt DA, Jr, Jeyendran RS. 1987. Rouleaux formation by spermatozoa in the naked-tail armadillo, Cabassous unicinctus. J Reprod Fertil 79:153–158.

Higginson DM, Pitnick S. 2011. Evolution of intra-ejaculate sperm interactions: Do sperm cooperate? Biol Rev Cam Philos Soc 86:249–270.

Immler S, Moore HD, Breed WG, Birkhead TR. 2007. By hook or by crook? Morphometry, competition and cooperation in rodent sperm. PLoS ONE 2:e170.

Immler S. 2008. Sperm competition and sperm cooperation: The potential role of diploid and haploid expression. Reproduction 135:275–283.

Jervis KM, Robaire B. 2001. Dynamic changes in gene expression along the rat epididymis. Biol Reprod 65:696–703.

Johnston DS, Jelinsky SA, Bang HJ, DiCandeloro P, Wilson E, Kopf GS, Turner TT. 2005. The mouse epididymal transcriptome: Transcriptional profiling of segmental gene expression in the epididymis. Biol Reprod 73:404–413.

Johnston SD, Smith B, Pyne M, Stenzel D, Holt WV. 2007. One-sided ejaculation of echidna sperm bundles. Am Nat 170:E162–E164.

Jones RC, Dacheux JL, Nixon B, Ecroyd HW. 2007. Role of the epididymis in sperm competition. Asian J Androl 9:493–499.

Jones RC, Ecroyd H, Dacheux JL, Nixon B. 2009. Testicular descent, sperm maturation and capacitation. Lessons from our most distant relatives, the monotremes. Reprod Fertil Dev 21:992–1001.

Krause WJ, Cutts JH. 1979. Pairing of spermatozoa in the epididymis of the opossum (Didelphis virginiana): A scanning electron microscopic study. Arch Histol Jpn 42:181–190.

Martan J, Adams CS, Perkins BL. 1970. Epididymal spermatozoa of two species of squirrels. J Mammal 51:376–378.

Martan J, Hruban Z. 1970. Unusual spermatozoan formations in the epididymis of the flying squirrel (Glaucomys volans). J Reprod Fert 21:167–170.

Martan J, Shepherd BA. 1973. Spermatozoa in rouleaux in the female guinea pig genital tract. Anat Rec 175:625–629.

Martan J. 1970. Epididymal spermatozoa in chinchilla. Anat Rec 166:345.

Mital P, Hinton BT, Dufour JM. 2011. The blood-testis and blood-epididymis barriers are more than just their tight junctions. Biol Reprod; 84:851–858.

Monclus MA, Cesari A, Cabrillana ME, Boarelli PV, Vincenti AE, Burgos MH, Fornes MW. 2007. Sperm rosette: Assembling during epididymal transit, in vitro disassemble, and oligosaccharide participation in the linkage material. Anat Rec (Hoboken) 290:814–824.

Monclus MA, Cesari A, Cabrillana ME, Saez Lancellotti TE, Rensetti DE, Clementi MA, Boarelli PV, Vincenti AE, Fornes MW. 2010. Protein fraction isolated from epididymal fluid reassociates sperm in vitro: Possible role of serpins in rat rosettes assembly. Mol Reprod Dev 77:410–419.

Moore H, Dvoráková K, Jenkins N, Breed W. 2002. Exceptional sperm cooperation in the wood mouse. Nature 418:174–177.

Moore HD, Taggart DA. 1995. Sperm pairing in the opossum increases the efficiency of sperm movement in a viscous environment. Biol Reprod 52:947–953.

Moore T, Moore HD. 2002. Marsupial sperm pairing: A case of ''Sticky'' green beards. Trends Ecol Evol 17:112–113.

Nixon B, Ecroyd HW, Dacheux JL, Jones RC. 2011. Monotremes provide a key to understanding the evolutionary significance of epididymal sperm maturation. J Androl 32:665–671.

Orsi AM, De Melo VR, Gonçalves RP, Ferreira AL. 1981. Subcellular structure of the luminal content of the cauda epididymidis of the opossum (Didelphis azarae). J Submicrosc Cytol 13:569–573.

Pickett BW, Burwash LD, Voss JL, Back DG. 1975. Effect of seminal extenders on equine fertility. J Anim Sci 40:1136–1143.

Phillips DM. 1970. Ultrostructure of spermatozoa of the wooly opossu Caluromys philander. J Ultrastruc Res 33:381–397.

Phillips DM, Bedford JM. 1987. Sperm-sperm associations in the loris epididymis. Gamete Res 18:17–25.

Pitnick S, Hosken DJ, Birkhead TR. 2009a. Sperm morphological diversity. In: Birkhead TR, Hosken DJ, Pitnick S, editors. Sperm biology, an evolutionary perspective. San Diego Academic Press. pp 130–131.

Pitnick S, Hosken DJ, Birkhead TR. 2009b. Sperm morphological diversity. In: Birkhead TR, Hosken DJ, Pitnick S, editors. Sperm biology, an evolutionary perspective. San Diego Academic Press. pp 132–133.

Pizzari T, Parker GA. 2009. Sperm competiton and sperm phenotype. In: Birkhead TR, Hosken DJ, Pitnick S, editors. Sperm biology, an evolutionary perspective. San Diego Academic Press. pp 258–259.

Pizzari Foster TKR. 2008. Sperm sociality: Cooperation, altruism, and spite. PLoS Biol 6:e130.

Radigue C, Soufir JC, Couvillers ML, Dantec M, Folliot R. 1988. Early effects of gossypol on the testis and epididymis in the rat. Reprod Nutr Dev 28:1329–1338.

Restrepo B, Cardona-Maya W. 2012. Antisperm antibodies and fertility association. Actas Urol Esp 37:571–578.

Robaire B, Hermo L. 1988. Efferent ducts, epididymis and vas deferens: Structure, functions and their regulation. In: Knobil E, Neill J, editors. The physiology of reproduction. New York: Raven Press. pp 999–1080.

Robaire B, Viger RS. 1995. Regulation of epididymal epithelial cell functions. Biol Reprod 52:226–236.

Rodger JC, Bedford JM. 1982. Separation of sperm pairs and sperm-egg interaction in the opossum, Didelphis virginiana. J Reprod Fertil 64:171–179.

Rose NR, Hjort T, Rumke P, Harper MJK, Vyazov O. 1976. Techniques for detection of iso and auto-antibodies to human spermatozoa. Clin Exp Immun 23:175–199.

Roy N, Majumder GC. 1989. Purification and characterization of an anti-sticking factor from goat epididymal plasma that inhibits sperm-glass and sperm-sperm adhesions. Biochim Biophys Acta 991:114–122.

Roy D, Dey S, Majumder GC, Bhattacharyya D. 2014. Occurrence of novel Cu(2+)-dependent sialic acid-specific lectin, on the outer surface of mature caprine spermatozoa. Glycoconj J 31:281–288.

Sandera M, Albrecht T, Stopka P. 2013. Variation in apical hook length reflects the intensity of sperm competition in murine rodents. PLoS ONE 8:e68427.

Senger PL, Saacke RG. 1976. Serum-induced head-to-head agglutination of bovine spermatozoa. J Reprod Fertil 47:215–219.

Shackelford TK, Goetz AT. 2007. Adaptation to sperm competition in humans. Curr Dir Psych Sci 16:47–50.

Simeone FA, Young WC. 1931. A study of the function of the epididymis. IV. The fate of non-ejaculated spermatozoa in the genital tract of the male Guinea pig. J Exp Biol 8:163–175.

Suarez SS. 1988. Hamster sperm motility transformation during development of hyperactivation in vitro and epididymal maturation. Gamete Res 19:51–65.

Sullivan R, Saez F, Girouard J, Frenette G. 2005. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol Dis 35:1–10.

Taggart DA, Johnson JL, OBrien HP, Moore HD. 1993. Why do spermatozoa of American marsupials form pairs? A clue from the analysis of sperm-pairing in the epididymis of the grey short-tailed opossum, Monodelphis domestica. Anat Rec 236:465–478.

Temple-Smith PD, Bedford JM. 1980. Sperm maturation and the formation of sperm pairs in the epididymis of the opossum, Didelphis virginiana. J Exp Zool 214:161–171.

Temple-Smith PD. 1987. Sperm structure and marsupial phylogeny. In: Archer M, editor. Possum and opossum: Studies in evolution. Sydney: Surrey Beatty and Sons. pp 273–277.

Toshimori K. 2003. Biology of spermatozoa maturation: An overview with an introduction to this issue. Microsc Res Tech 61:1–6.

Tung KS, Okada A, Yanagimachi R. 1980. Sperm autoantigens and fertilization. I. Effects of antisperm autoantibodies on Rouleaux formation, viability, and acrosome reaction of guinea pig spermatozoa. Biol Reprod 23:877–886.

Usselman MC, Cone RA. 1983. Rat sperm are mechanically immobilized in the caudal epididymis by ''immobilin,'' a high molecular weight glycoprotein. Biol Reprod 29:1241–1253.

Varea-Sánchez M, Gómez Montoto L, Tourmente M, Roldan ER. 2014. Postcopulatory sexual selection results in spermatozoa with more uniform head and flagellum sizes in rodents. PLoS ONE 9:e108148.

Veeramachaneni DN, Moeller CL, Sawyer HR. 2006. Sperm morphology in stallions: Ultrastructure as a functional and diagnostic tool. Vet Clin North Am Equine Pract 22:683–692.

Verberckmoes S, van Soom A, de Pauw I, Dewulf J, Rijsselaere T, de Kruif A. 2004. Effect of whole blood and serum on bovine sperm quality and in vitro fertilization capacity. Theriogenology 61:25–33.

Visconti PE, Kopf GS. 1998. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod 59:1–6.

Williamson BR, Shepherd BA, Martan J. 1980. Fertility of spermatozoa from the excurrent ducts of the guinea-pig. J Reprod Fertil 59:515–517.

World Health Organization. 2010. WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th edition. Geneva, Switzerland: WHO Press, World Health Organization. pp 34–36.

Yanagimachi R. 1982. In vitro sperm capacitation and fertilization of golden hamster egg in a chemical defines medium. In: ''In vitro fertilization and embryo transfer'' (Hafez ESE, Semm K, editors). Lancaster UK: MTP Press LTD. pp 65–76.

Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill J, editors. The physiology of reproduction. New York: Raven Press. pp 189–317.

Yanagimachi R, Mahi CA. 1976. Sperm acrosome reaction and fertilization in the guinea-pig: A study in vivo. J Reprod Fertil 46:49–54.

Yanagimachi R, Kamiguchi Y, Sugawara S, Mikamo K. 1983. Gametes and fertilization in the Chinese hamster. Gamete Res 8:97–117.

Yang DH, McMillan AG, Standley NT, Shannon P, Xu ZZ. 2012. Extracellular calcium is involved in egg yolk-induced head-to-head agglutination of bull sperm. Theriogenology 78:1476–1486.