

Multifunctional glycoprotein DEFB126—a curious story of defensin-clad spermatozoa

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Abstract | During maturation, the surface of mammalian spermatozoa undergoes dramatic changes leading to the acquisition of properties vital for survival and performance in the female reproductive tract. A prominent change is the addition to the sperm surface of an atypical β -defensin polypeptide. In primates, the β -defensin DEFB126 becomes adsorbed to the entire sperm surface as spermatozoa move through the epididymal duct. DEFB126 has a conserved β -defensin core and a unique long glycosylated peptide tail. The carbohydrates of this domain contribute substantially to the sperm glycocalyx. DEFB126 is critical for efficient transport of sperm in the female reproductive tract, preventing their recognition by the female immune system, and might facilitate the delivery of capacitated sperm to the site of fertilization. A newly discovered dinucleotide deletion in the human *DEFB126* gene is unusually common in diverse populations and results in a null allele. Predictably, men who are homozygous for the deletion produce sperm with an altered glycocalyx and impaired function, and have reduced fertility. Insights into the biology of DEFB126 are contributing to a better understanding of reproductive fitness in humans, as well as the development of diagnostics and therapeutics for male infertility.

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Introduction

When moving through the female reproductive tract, fertilizing sperm must traverse an assortment of mucosal fluids and extracellular matrices. While they move smoothly across some epithelial surfaces, they are required to bind reversibly with others, and all the while they must evade phagocytes, complement proteins, and antibodies. Successful spermatozoa, therefore, must possess surface properties particularly tailored for this difficult journey. Mammalian sperm are enclosed in an extensive glycocalyx—a dense scaffold of surface-associated oligosaccharides that serve as the primary interface between sperm and the multiple microenvironments of the female tract.^{1,2}

Immediately after spermatogenesis, mature mammalian sperm possess only a rudimentary glycocalyx,² but they undergo extensive surface remodelling as they move through the epididymis over the next 3–12 days (depending on species).³ Most notably, an array of complex sugars attach to the proteins and lipids of the plasma membrane.² Concomitant with these surface changes, sperm acquire motility and the ability to achieve fertilization. Specific components of the glycocalyx have been shown to be critical for sperm protection, penetration of the cumulus matrix, recognition of the zona pellucida and oolemma, and fusion of the sperm with the egg plasma membrane.⁴ These findings have led to the hypothesis

that formation of the sperm glycocalyx in the male efferent duct is critical for sperm survival and proper functioning in the female tract.² Furthermore, the intimate association of glycoconjugates with sperm seems to be a conserved feature of animals that reproduce via internal fertilization (Box 1).

DEFB126 orthologs contribute to the sperm glycocalyx in many species, including human, monkey, mouse and rat. In primates, the molecule is named DEFB126, whereas its orthologs in rodents carry the name Defb22. In the cynomolgus monkey (*Macaca fascicularis*), DEFB126 is a major component of the sperm glycocalyx and is adsorbed to the surface of sperm during epididymal transit (Figure 1). Synthesis of DEFB126 is limited to principal cells of epididymal epithelium, with maximal expression observed in the distal corpus and proximal cauda.^{5,6} This pattern of expression seems to be conserved among orthologs, as observed for DEFB126 in the human,⁷ and Defb22 in the rat^{8,9} and mouse.^{8,10} This glycoconjugate forms a continuous covering over the entire surface of macaque sperm that varies little in density,^{5,11} a pattern that is shared by the mouse (Figure 2).¹⁰ Likewise, orthologs appear similarly distributed on sperm from human¹² and rat,¹³ although in these species the coating appears to be less uniform.

Recently, a polymorphism in the human *DEFB126* gene that disables the synthesis of DEFB126 has been reported to result in major alterations in the composition of the glycocalyx of human sperm.¹² Sperm that are not coated with DEFB126 display reduced performance in reaching the egg, which equates to reduced fertility.¹²

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Competing interests

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Key points

- β -defensin DEFB126 is adsorbed to the sperm surface during epididymal maturation
- DEFB126 has an atypical β -defensin structure, possessing a highly glycosylated peptide tail that contributes substantially to the sperm glycocalyx
- The glycosylated tail of DEFB126 confers properties to sperm shown in macaques to be important for transport in the female reproductive tract
- DEFB126 protects macaque sperm from immune surveillance in the female reproductive tract
- A mutation in the human *DEFB126* gene that disables the synthesis of DEFB126 results in major alterations in the glycocalyx of sperm, poor sperm performance in cervical mucus-like viscous gels, and reduced fertility

Box 1 | Glycoconjugates and internal fertilization

Across phyla, glycoconjugates associated with spermatozoa, whether derived from the male or female, are critical for maintaining sperm viability and function in the female tract.⁸⁰ Secreted glycoproteins that coat sperm have been demonstrated to preserve sperm integrity and extend sperm longevity in many species, including salamander,⁸¹ fish,⁸² crab,⁸³ and *Drosophila*.^{84–86} In poeciliid fish, sperm survive in the female tract for several months and through successive ovulations as a consequence of the carbohydrate secretions of the efferent ducts of the testes that are transferred with sperm to the female during mating.⁸² In general, however, sperm from invertebrates and lower vertebrates undergo limited migration through the female tract; instead these sperm are typically deposited within the tract already packed in a dense extracellular matrix of carbohydrates, which maintains sperm in a relatively quiescent state until approached by an oocyte. By contrast, birds and mammals have highly motile sperm that migrate much of the length of the female tract, and carry glycoconjugates with them in the form of a highly developed glycocalyx.^{2,11,16,87,88} In mammals, carbohydrates tether sperm to epithelial cells of the oviductal isthmus, establishing a reservoir of sperm that are preserved for a period of days to months (depending on species) until the time of ovulation.³⁵ Similarly, in poultry, the sperm glycocalyx is necessary for population of sperm storage tubules, which are the functional equivalent of the oviductal reservoir in mammals.^{89–91} In mammals, sialylated glycoconjugates of the glycocalyx potentially also contribute to the longevity of sperm in the female reproductive tract by masking the otherwise highly antigenic sperm surface from immunologic detection.^{92–95} Collectively, these observations suggest a conserved strategy of internal fertilization—the use of glycoconjugates to maintain sperm viability during extended times of residence within the female.

In this Review, we provide an overview of the functions of DEFB126, including recent investigations of its orthologs in mice, monkeys, and humans, and we discuss the importance of this unusual molecule in sperm function.

DEFB126

DEFB126 is a molecule with distinctive structural features, which include a cysteine-rich canonical β -defensin core motif and a highly glycosylated carboxyl terminus. Originally termed epididymis-specific protein ESP13.2 in the macaque,^{5,6} it was soon discovered that the core of this highly expressed glycopeptide contained the six cysteine residues characteristic of β -defensins,¹⁴ a family of peptides instrumental to the innate immune system (Box 2). Subsequently, ESP13.2 was reclassified as a β -defensin and named DEFB126 (Defb22 in rodents). With an apparent molecular mass of 32–35 kDa, DEFB126 is unusually large for a β -defensin and can be distinguished from other members of the family by a unique 60 amino acid carboxyl terminus adjacent to the β -defensin core region.⁵ This extended carboxyl tail has 20 candidate sites (serine and threonine residues) for

O-linked glycosylation and one unpaired cysteine that might mediate dimerization.¹¹ Treatment of macaque sperm with O-glycanase reduces the apparent molecular weight of DEFB126 to 10 kDa, consistent with its deduced amino acid sequence and more in line with the size of a typical β -defensin molecule. These data demonstrate that carbohydrates are responsible for the majority of the observed molecular mass of DEFB126.¹⁴

Immunolocalization studies using fluorescence and transmission electron microscopy show that DEFB126 in the macaque,⁵ and Defb22 in the mouse,¹⁰ is uniformly distributed over the entire sperm surface (Figure 2).⁵ DEFB126 is tightly adhered to the sperm surface, resisting removal by centrifugation through density-gradient solutions and high salt conditions.^{5,15} DEFB126 appears to interact with the lipid membrane as a covalently linked dimer.^{5,16} Similarly, during isolation of Defb22 from rat sperm, the majority of protein was found in the lipid fraction as a disulfide cross-linked homodimer.¹³ As such, it has been proposed that Defb22 embeds firmly in the lipid bilayer via sequences of hydrophobic amino acids associated with the defensin-like core. DEFB126 in the macaque is readily shed from the entire surface of sperm under conditions that support capacitation,¹⁵ a complex series of postejaculatory changes that sperm must undergo to acquire the capacity to bind and fertilize the egg.¹⁷ Immunogenicity and differential lectin mapping studies of sperm surface carbohydrates provide evidence that DEFB126 in the macaque,^{11,14} and its ortholog Defb22 in the mouse,¹⁰ are major components of the sperm glycocalyx in these species, and seem to exclusively occupy the exposed or outer reaches of the glycocalyx.

DEFB126 and sperm transport**Interaction of sperm with the cervical mucus**

Primates, rabbits and ruminants are the only mammalian species that ejaculate sperm into the vagina rather than into the cervix.¹⁸ In these species, the cervical mucus plays a vital role in facilitating sperm entry and in routing quality sperm to the uterus. Initially, sperm are drawn into cervix by pressure gradients generated by muscular contractions of the female reproductive tract.¹⁹ This passive phase of sperm transport is short-lived, however, and subsequent movement of sperm through the cervical canal requires that sperm propel themselves by vigorous flagellar beating.²⁰ Sperm swimming in cervical mucus must penetrate its glycoprotein-dominated microstructure.^{21–24} Therefore, efficient swimming depends largely on the surface properties of sperm.²⁵

By the time of ejaculation, macaque sperm have been densely and uniformly coated with DEFB126.⁵ The sperm remain coated as they migrate into and out of mucus both *in vitro* and following natural mating (Figure 3).²⁶ In fact, virtually all viable sperm (>99%) recovered from the uterus of mated female macaques still possess a surface coating of DEFB126.¹⁵ Removal of the DEFB126 glycocalyx inhibits the numbers of sperm that can traverse periovulatory cervical mucus *in vitro* by over 80%.²⁶ Rather than the characteristic smooth, continuous

movement of sperm through periovalary mucus in relatively linear paths, sperm lacking DEFB126 seem to struggle, exhibiting frequent interruptions in progressive movement and a more tortuous swimming path, as if probing for regions of mucus that could be more easily traversed. The ability of sperm to penetrate mucus can be completely restored when soluble DEFB126 is added to the sperm surface.²⁶

Among the oligosaccharides attached to DEFB126, those that are negatively charged seem to be crucial for sperm to swim efficiently in cervical mucus in macaques.²⁶ Treatment of sperm with neuraminidase, which removes terminal sialic acid groups, eliminates most of the negative charge from DEFB126^{11,14} and therefore reduces the surface charge of sperm.¹¹ Neuraminidase treatment dramatically inhibits sperm mucus penetration *in vitro*, comparable in magnitude to removal of DEFB126 or treatment of sperm with anti-DEFB126 antibodies.²⁶ Furthermore, cervical mucus penetration can be blocked in a dose-dependent manner by addition of charge-neutralizing poly-L-lysine.²⁶ The precise mechanism by which the surface charge of sperm promotes efficient mucus penetration is not known, but we speculate that the 'shell' of charge provided by DEFB126 is necessary to minimize the interaction of sperm with the negatively charged cervical mucus.^{27,28} When both sperm and mucus are negatively charged, a mutual repulsion occurs, enabling sperm to slide through channels of mucus with minimal resistance.

Immunoprotection in the endocervix and uterus

The female reproductive tract is highly immunocompetent near the time of ovulation. Under the influence of oestrogen, the mucosal secretion of antibodies increases, reaching a maximum during the late follicular phase.^{29,30} Similarly, leukocyte response reaches peak sensitivity during late follicular phase owing to the oestrogen-enhanced production of cytokines and chemokines by reproductive tract epithelium.³¹ As a result, the female reproductive tract is in a state of readiness for the purpose of combating microbial pathogens that can gain access during copulation. This preparedness involves several immunological barriers to sperm, which are in essence allogeneic foreign cells. First, periovalary cervical mucus contains IgA, and exudates of the vagina and uterus contain elevated levels of IgG.²⁹ Under normal circumstances, a portion of immunoglobulins target sperm,³² and in some women these antisperm antibodies can impair fertility.³³ Furthermore, insemination induces a number of inflammatory responses, chief of which is leukocyte migration.³⁴ 30 min to 2 h after semen deposition (depending on species), leukocytes infiltrate the mucosa and luminal spaces of the endocervix and uterus in numbers far exceeding that of spermatozoa.¹⁸ How fertilizing sperm evade immunodetection and elimination is a subject of some debate, but several lines of evidence suggest that sialylated glycoconjugates of the sperm glycocalyx contribute to sperm protection by masking the otherwise highly antigenic surface (Box 1).

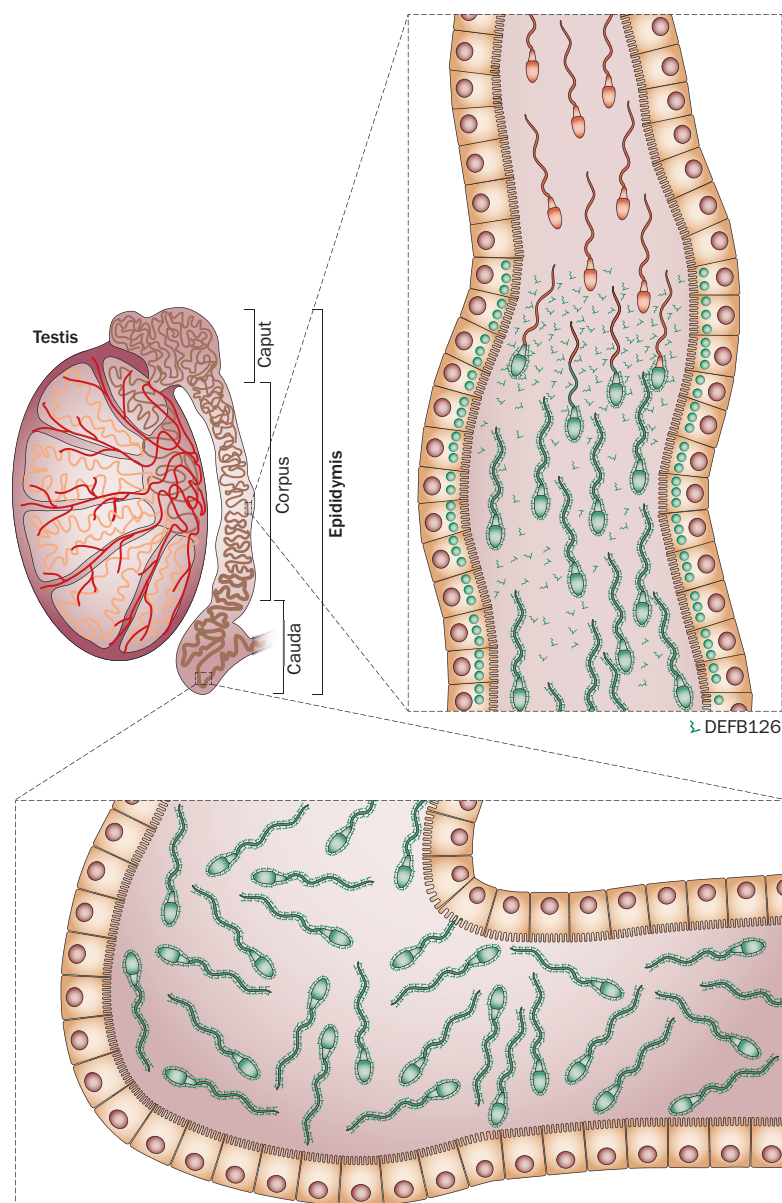


Figure 1 | DEFB126 is adsorbed to sperm during epididymal transit. DEFB126 is secreted by principal cells of the distal corpus. As sperm move through the corpus region of the epididymis, they are coated with DEFB126 from the tip of the head to the end of the terminal piece of the flagellum. Sperm are stored in the cauda under conditions that stabilize DEFB126 on the sperm surface.

Immunoprotection seems to be conferred to sperm by the sialylated oligosaccharides on DEFB126, in the macaque. Experimentally, this was determined by eliciting an antisperm antibody response to aldehyde-fixed sperm in rabbits, and comparing whole (control) sperm to sperm where either DEFB126 was removed or its carbohydrates enzymatically modified.¹⁴ Antisera from rabbits immunized with control sperm showed a strong reaction only to DEFB126 on western blots of whole sperm. By contrast, additional sperm proteins were recognized in the antisera from rabbits immunized with sperm with DEFB126 removed.¹⁴ This same pattern of antisera recognition was also demonstrated when sialic acid was removed from sperm with neuraminidase prior

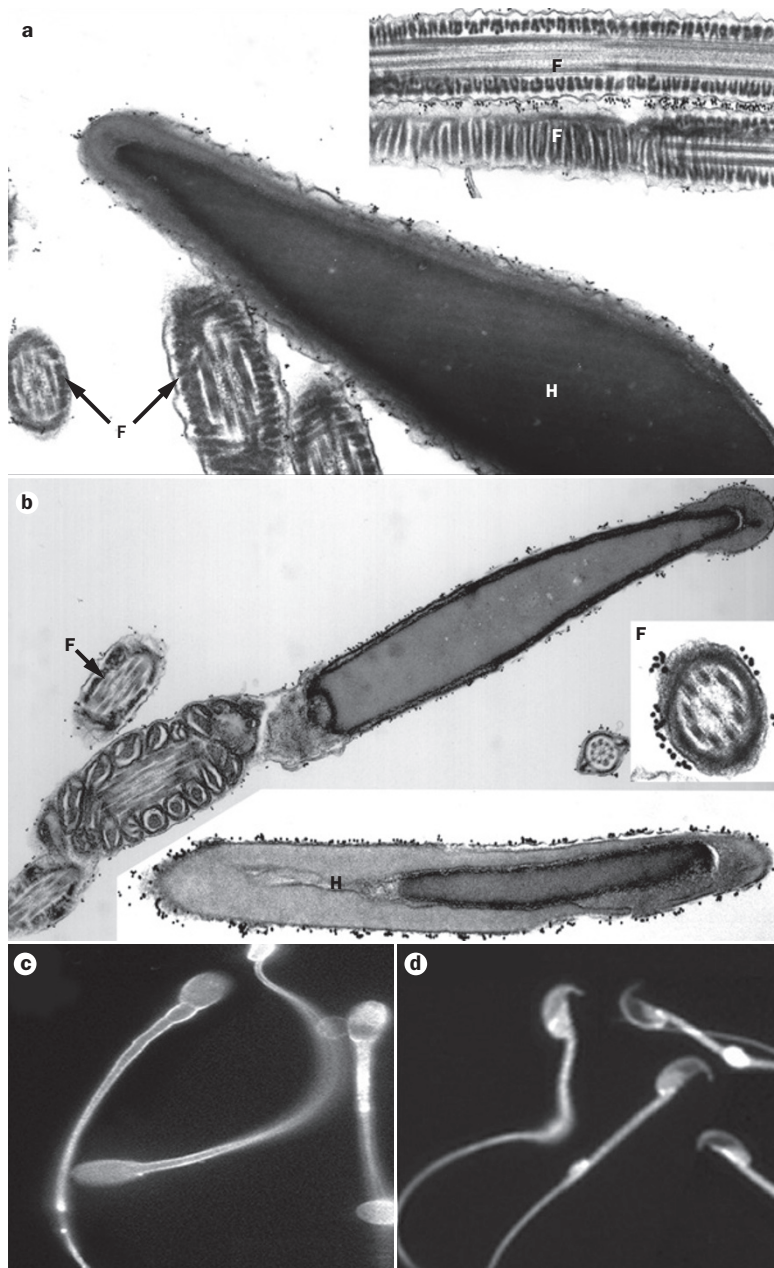


Figure 2 | Electron micrographs show immunogold localization of DEF126. **a, c** | An antibody specific to DEF126 recognizes the glycosylated defensin on washed caudal macaque sperm, revealing even distribution along the plasma membrane of the entire sperm. **b, d** | Similarly, even distribution of Defb22 is observed in mouse sperm. F marks flagellum; H marks sperm head. Permissions obtained from *Society for the Study of Reproduction* © Yudin, A. I. et al. *Biol. Reprod.* **69**, 1118–1128 (2003) and from *Society for Reproduction and Fertility* © Yudin, A. I. et al. *Reproduction* **136**, 735–765 (2008).

to immunization. Moreover, the staining of live sperm with polyclonal antibodies specific to three different integral membrane proteins was observed only when sperm were first treated to remove DEF126 or treated with neuraminidase.¹⁴ Finally, antibodies against PH20 (also called SPAM 1, a sperm surface hyaluronidase) recognize its target antigen on the head of both macaque and murine sperm only when DEF126 (or the mouse ortholog Defb22) is removed (Figure 4). Collectively, these

experiments suggest that the DEF126-containing glyco- calyx effectively covers up other protein components on the sperm surface, making sperm essentially invisible to immune surveillance.

Sperm–oviduct interaction

In most mammals, the formation of a reservoir of sperm in the oviduct seems to be critical for successful fertilization. During ascent of the female tract, only a small fraction of ejaculated sperm successfully migrates to the isthmic region of the oviduct. The isthmic sperm reservoir appears to be formed by selective binding of fertilization-competent sperm to epithelial cells of the oviduct.³⁵ Scanning electron microscopy has revealed that sperm bind to oviductal epithelial cells (OECs) via the plasma membrane overlying the acrosome.^{36,37} Although there are many differences between species in the specifics of how sperm engage OECs, in general, initiation of sperm–OEC binding requires noncapacitated sperm and carbohydrate recognition.^{38,39} Depending on the species, sperm can be stored for hours to many days in the oviduct until ovulation.^{40,41}

Macaque sperm exhibit DEF126-dependent binding to oviductal epithelia. Sperm bound to epithelium in the isthmic half of the periovarian oviducts resist displacement from fluid shear forces. However, the sperm–OEC interaction is reduced by approximately 80% following either induction of capacitation to remove DEF126 from sperm, treatment with anti-DEF126 immunoglobulins, or removal of sialic acid from sperm surface oligosaccharides.¹⁶ Addition of soluble DEF126 to the sperm results in full recovery of sperm–OEC binding. Purified soluble DEF126 binds specifically to secretory cells of the oviductal epithelium *in situ* and coincides with the location of sperm head attachment on oviductal explants (Figure 5).¹⁶ Thus, DEF126 seems to provide a means by which sperm reaching the oviduct can dock and conserve resources until arrival of the oocyte.

Sperm release from oviductal reservoirs

At the time of ovulation, a small number of sperm can be found at the site of fertilization—known as the ampulla or the ampullar–isthmic junction of the oviduct. In rodents, the ratio of the number of sperm to eggs in the ampulla approaches unity.¹⁸ This fine-tuning is accomplished by the gradual release of fertilization-competent sperm from the oviductal isthmus.⁴² The importance of the highly regulated release of sperm is probably twofold, preventing polyspermy and delivering just enough capacitated sperm to the eggs before they age.¹⁸

In mammals, the mechanisms that underlie the timed release of sperm from oviductal stores are complex and incompletely understood, but seem to involve dramatic shifts in the composition of oviductal fluid that coincide with ovulation. Changes in levels of inorganic ions and pH,^{43,44} energy substrates,^{45,46} periovarian hormones,^{42,47} and glycosaminoglycans,^{48,49} have been shown to induce or promote capacitation *in vitro* in a variety of species.⁵⁰ Capacitated sperm are able to escape the oviductal reservoirs by shedding molecules that tether

them to oviductal cells and through the development of hyperactivated motility, a pattern of vigorous flagellar beating, which allows sperm to break away from cellular attachments and mucus-laden oviductal crypts.³⁸ Release of sperm might be further aided by oviduct secretion of sulphated glycoconjugates and reduced glutathione, which can in turn reduce disulfide bonds on the sperm surface.⁵¹ In cattle, the subsequent increase of sulphhydryl groups on the sperm surface is associated with loss of sperm adhesion to *in vitro* cultured oviductal epithelium.⁵²

The bicarbonate and glucose composition of primate oviductal fluid at the time of ovulation is sufficient to trigger the release of DEF126 from macaque sperm.⁵³ A sudden increase in bicarbonate secretion in the oviduct accounts for a shift in mucosal fluid pH from approximately 7.2 during the late follicular phase to 7.8 at ovulation in macaques.⁵⁴ Also corresponding with midcycle events, a multifold decrease in glucose concentration in oviductal fluid has been observed in humans.^{45,46} These potentially coinciding changes in primate mucosal fluid conditions have synergistic effects on sperm; while bicarbonate drives intracellular signalling processes and changes in sperm membrane composition that are required for sperm capacitation,¹⁷ the reduced availability of glucose results in less metabolic acid, enabling the sperm cytosol to alkalinize, thereby potentiating the effects of bicarbonate on capacitation.^{55,56} In medium that simulates primate periovulatory oviductal fluid, a comparable combination of elevated bicarbonate (and pH), and reduced glucose induces both the rapid release of DEF126 from the surface of macaque sperm and promotes capacitation.⁵³

Before ovulation, the fluid environment of the oviductal isthmus resembles that of the corpus and caudal epididymis (with respect to inorganic ions), and probably serves to maintain sperm in a quiescent state. As sperm move from caput to cauda, the epididymal milieu becomes more acidic as bicarbonate is efficiently reabsorbed in the proximal epididymis while proton ATPases and exchangers secrete hydrogen ions into the distal epididymis.^{57,58} Simultaneously, the concentration of calcium ions steadily drops in epididymal fluid from 0.85 mM to 0.25 mM⁵⁹ and potassium ion concentration increases twofold from 20 mM to 40 mM.^{59,60} Such extracellular ionic conditions have been shown to inhibit or delay capacitation *in vitro*¹⁷ and are very similar to those present in the oviduct during the follicular phase,⁴⁵ a time when sperm are stored in the oviductal reservoir.⁶¹ The ionic conditions of the epididymis might serve, in part, to stabilize sperm lipids and surface coats, including DEF126. The epididymis-like conditions in the oviduct reverse as ovulation approaches—potassium ion levels decrease,⁴⁵ calcium levels peak,⁶² bicarbonate levels rise and pH increases.^{45,54} These events coincide with the release of sperm from the reservoir in a capacitated state.⁶¹ Ionic conditions consistent with periovulatory oviductal milieu are supportive of capacitation *in vitro* in the macaque,⁵³ as well as in mice, rabbits, guinea pigs, hamsters, cattle, and humans.¹⁷

Box 2 | Defensins

Defensins are effector peptides involved in innate immunity. Now known to be widely distributed in the plant and animal kingdoms, they were first discovered 25 years ago in mammals based on their antimicrobial activity in phagocytic white cells and mucosal epithelial cells.^{96–98} Structurally, defensins are cationic peptides comprised of 29–42 amino acids including six conserved cysteines. The cysteine residues form three intramolecular disulfide bonds, which fold the peptide in an active conformation that confers resistance to protease cleavage. Most defensins exhibit broad antimicrobial activity against bacteria, fungi and some viruses. This activity is attributed to their ability to associate with lipid bilayers, and to form pores in the negatively charged membranes of infectious microbes. Some defensins also have properties similar to those of chemotactic cytokines, which result from binding to G-protein-coupled receptors. Defensins can be divided into three subfamilies (α , β , and θ) based on structural subtleties at the peptide and gene levels.⁹⁸ Homologs of human β -defensins have been identified in ruminants, marsupials, rodents, birds, fish, and horseshoe crabs. Approximately 40 β -defensin genes exist in the human genome, although data on peptide activity and biology are available for <10; most of these data are on β -defensins expressed by mucosal epithelial cells of the respiratory, digestive, and reproductive tracts.⁹⁹ Curiously, mRNAs encoding nearly half of all human β -defensins are found in the epididymis.^{73,100} Expression of β -defensin mRNA in the epididymis has been well-characterized in rodents^{8,100} and primates,⁷³ including humans,^{7,100} and has been demonstrated to be androgen dependent in primates.⁷³ It was initially assumed that β -defensins were involved in innate immune protection of the male reproductive tract but new studies indicate that at least a few of these defensins, including DEF126, have a role in reproductive physiology.⁷³

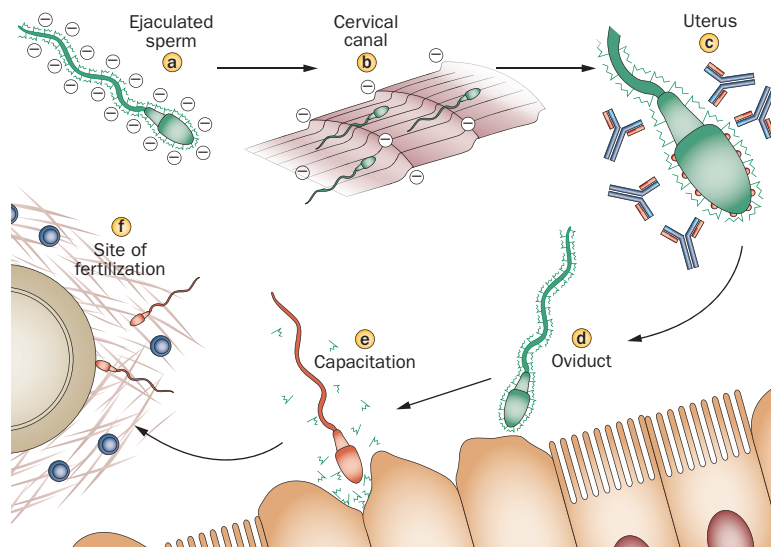


Figure 3 | DEF126 facilitates sperm transport. **a** | Ejaculated sperm is coated with DEF126 over its entire surface. **b** | This coating imparts a high negative charge that enables sperm to swim smoothly through the negatively charged cervical mucus. **c** | Sperm reaching the uterus retain their DEF126 coating, which might provide protection from surveillance and response by both the innate and adaptive immune systems of the female. **d** | Sperm reaching the oviduct remain coated with DEF126, which mediates attachment to the oviductal epithelium. Sperm are kept in a quiescent state until the time of ovulation. A reservoir of sperm is formed as more sperm migrate into the oviduct and bind to the epithelium. **e** | During ovulation, an elevation in pH of oviductal fluid trigger the release of DEF126 from the sperm surface, enabling sperm to escape the oviductal reservoir. **f** | Once free of DEF126 in the oviduct, sperm are able to migrate to the site of fertilization. Elevation in bicarbonate and reduction in glucose in the oviductal fluid drive the completion of capacitation. With surface components now unmasked, such as the hyaluronidase PH20 and receptors for the egg, sperm can penetrate the hyaluronan-rich cumulus matrix and bind to the zona pellucida of the oocyte.

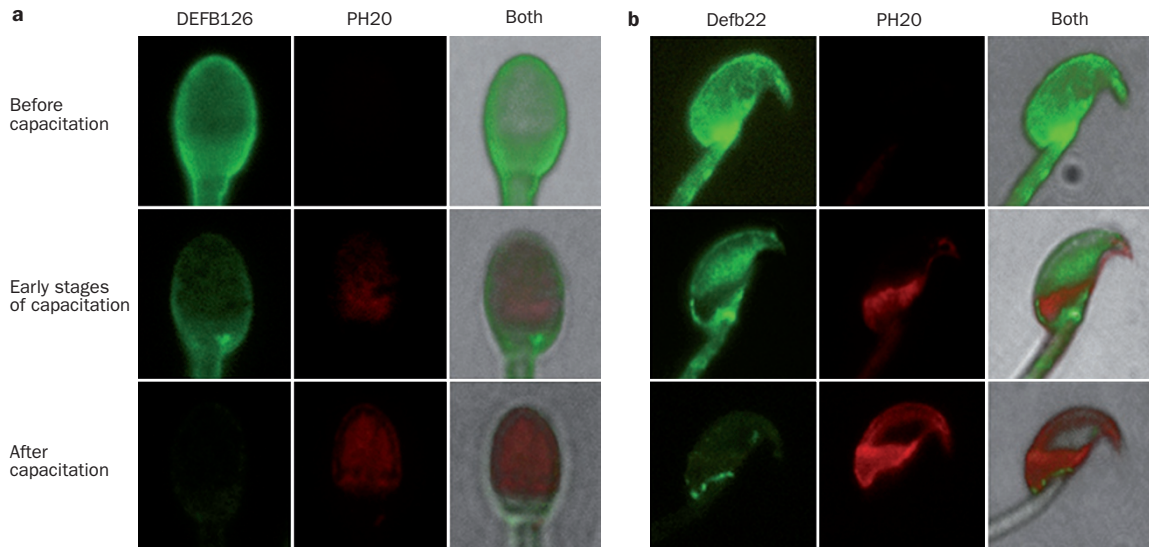


Figure 4 | The immunoprotective effect of DEFb126. Antibodies to sperm surface protein PH20 (a sperm surface hyaluronidase also called SPAM 1) cannot bind to the sperm surface until **a** | DEFb126 or **b** | Defb22 is released from sperm during capacitation.

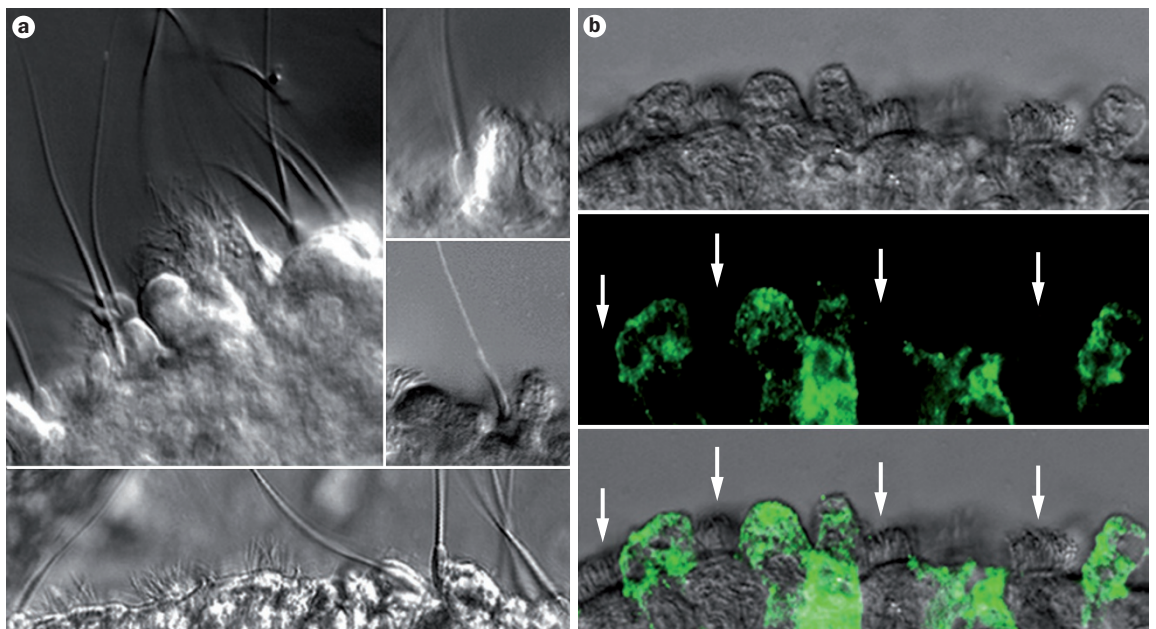


Figure 5 | DEFb126 binding to oviductal epithelium. **a** | Scanning laser confocal microscopy of oviductal explants using differential interference contrast reveals that sperm attach to and are embedded between secretory cells that protrude beyond the plane of the apical membrane. **b** | Intact oviducts were flushed *in situ* with soluble DEFb126. Confocal images of recovered epithelial explants show that DEFb126 binds to secretory cells and not ciliated cells (arrows). Permission obtained from Society for the Study of Reproduction © Tollner, T. L. *et al. Biol. Reprod.* **78**, 400–412 (2007).

Collectively, the implication of the *in vitro* and *in situ* oviductal studies in primates is that sperm are released from the oviductal reservoir by ovulation-specific changes in the lumen environment. These changes trigger the release of DEFb126 from sperm, without which sperm are no longer tethered to the oviductal epithelium and are then able to follow chemical and thermal cues towards the ampulla and the egg (Figure 3).⁶³ As pH and energy conditions gradually intensify as a result of increased midcycle secretory activity of oviductal epithelia,^{43,45,46}

more sperm are able to shed DEFb126,⁵³ enabling a steady supply of sperm at the site of fertilization.

Release of Defb22 from sperm in the mouse

The function of the mouse ortholog Defb22 in sperm behaviour in the female reproductive tract has not been determined in detail, but initial studies suggest that, like its primate counterpart, Defb22 is released from the surface of sperm after they reach the oviduct. Virtually all sperm recovered from the uteri of mated female mice

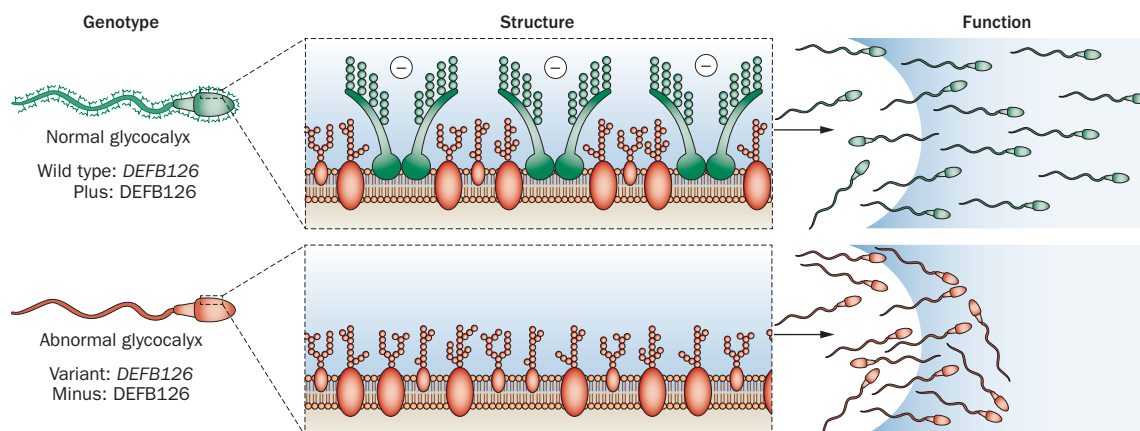


Figure 6 | *DEFB126* mutation, sperm glyco calyx structure and infertility. The oligosaccharide structure of the glyco calyx of sperm is significantly altered in men with the *del/del* genotype, compared to sperm from men possessing at least one wild type copy of the *DEFB126* gene (Genotype). The rich array of O-linked, negatively-charged oligosaccharides associated with dimeric *DEFB126* is absent in sperm from men with the *del/del* genotype (Structure). The loss of negative surface charge potentially explains why sperm from *del/del* men have difficulty penetrating gels that simulate cervical mucus (Function), and the decreased efficiency of sperm movement in cervical mucus might cause the reduced monthly probability of conception observed in men with the *del/del* genotype.

4 h after copulation retained *Defb22* over their entire surface.¹⁰ By contrast, some of the sperm recovered from oviductal fluid, and all sperm that had penetrated the cumulus, had lost *Defb22* from the head region.¹⁰ These observations suggest that mouse sperm retain *Defb22* for much of their journey along the female tract but release *Defb22* in the oviduct before interacting with the oocyte.

Mutation of the *DEFB126* gene in humans

A sequence variation in the *DEFB126* gene has recently been identified in the human population.¹² The variant is a two-nucleotide deletion (*del*) that results in a reading frame shift and an mRNA lacking an in-frame stop codon—a so-called nonstop mRNA.¹² Nonstop mRNAs are rapidly degraded in cells by mRNA surveillance mechanisms,^{64–68} are poorly translated,⁶⁹ and are effectively null in terms of function.^{66,67} Given the key biological functions of *DEFB126* discovered in the macaque, it was surprising to find that the *del* mutant allele was very common in population cohorts from China, Japan, Europe, and Africa (allele frequency 0.44–0.61).¹² These data indicate that approximately 20% of men worldwide are likely to be homozygous for the *DEFB126 del* allele.

Sperm from men who are homozygous for the deletion variant (*del/del*) seem normal with respect to common measures of male fertility potential, such as sperm count, percentage of sperm with vigorous motility, and percentage of sperm with normal form or shape.¹² However, the sperm from *del/del* men have significantly fewer O-linked oligosaccharides on their surface compared to sperm from wild-type males, indicating that the sperm glyco calyx is abnormal.¹² Furthermore, the sperm from *del/del* men have markedly impaired mobility in hyaluronic acid gels, an *in vitro* surrogate for cervical mucus.¹² Notably, an analysis of *DEFB126* genotype and reproductive success in a cohort of young, newly-married Chinese couples revealed that couples in which the male partner had a *del/del* genotype were only 60%

as likely to get pregnant over a 2-year period as couples where the men had either wild type (*wt/wt*) or heterozygous (*wt/del*) genotypes. This translated into a 30% reduction in the rate of live births per month.¹²

Based on these data, the *DEFB126* mutation is proposed to impair the mobility of sperm in cervical mucus and thereby decrease the likelihood of fertilization (Figure 6). Of course, the *DEFB126* mutation might affect fertility in other ways, such as reduced sperm function in the upper female tract or vulnerability to reproductive tract infections (given that *DEFB126* is a defensin), but these possibilities have yet to be explored.

Why might a deleterious allele be at such high frequency in the population? Analysis of *DEFB126* genotype data showed a small but statistically significant increase in frequency of heterozygotes compared to that predicted by the Hardy–Weinberg equilibrium, suggesting a possible heterozygous advantage.¹² Another possible explanation for the high variant allele frequency is antagonistic pleiotropy—that is, *DEFB126* might be responsible for multiple traits where one or more traits (as yet unknown) are beneficial to fitness, but another (reduced fertility) is detrimental.⁷⁰ This enigma remains unresolved, but is important to address experimentally, because the explanation might provide new insights into *DEFB126* biology.

The *DEFB126* mutation and its biological consequences have implications for the diagnosis and treatment of male infertility. Based on the published data, men with a *DEFB126 del/del* genotype produce sperm that, although apparently normal on conventional assessment, are dysfunctional in the female reproductive tract.¹² By establishing this genotype early in the evaluation of infertility, clinicians can justify, when appropriate, rapid progression to assisted reproductive techniques such as intrauterine insemination (IUI) and *in vitro* fertilization (IVF), thus saving couples the time and expense of a protracted workup. A limiting factor in the use of

Box 3 | Antimicrobial peptides in reproduction

Sexual reproduction and innate immunity are tightly linked throughout nature. Antimicrobial peptides, including linear (α -helical peptides) and cyclic (cysteine-containing peptides; β -hairpins and defensins) molecules, have been identified at high concentrations in the reproductive tissues of plants,^{74,101} invertebrates,^{84,86,102,103} lower vertebrates,^{75,76,82} and mammals.^{73,79,100} In nonmammalian vertebrates, antimicrobial peptides are often most highly expressed in male reproductive tissues relative to other tissues and are typically associated with compartments containing sperm.^{75,76,104} For example, in grouper fish the expression of β -defensins is much higher in the male than the female.⁷⁶ Only female fish that are hormonally modified to produce testes express large quantities of β -defensins, suggesting that evolutionarily speaking, males provide most of the antimicrobial peptides required for reproduction. In mammals, some defensins have taken on novel roles in the male. Similarly to DEF126 and its orthologs, Bin1b¹⁰⁵ and Defb15¹⁰⁶ in the rat are epididymal β -defensins that bind to sperm. Both Bin1b and Defb15 promote the acquisition of sperm motility during maturation.^{105,106} A transfer of antimicrobial peptides from male to female might be a conserved feature of internal reproduction. The delivery of male-derived peptides to the female during mating has been demonstrated in invertebrates,¹⁰³ including crabs,¹⁰² and *Drosophila*,⁸⁶ and in some cases these peptides provide antimicrobial protection to sperm in both the male and female tracts.^{86,102,103} This phenomenon has been less studied in vertebrates, with the delivery of DEF126 and its orthologs by sperm into the female tract potentially being the first reported examples in higher species.^{10,16} In both *Drosophila* and bed bugs, there is evidence that male-derived α -helical peptides (cecropins) are transferred at mating and reduce bacteria in the female tract.¹⁰⁷ These findings support the supposition that defensins and other antimicrobial peptides associated with sperm and male secretions aid the females' ability to clear microbes introduced during mating and might provide an environment less hostile to sperm.^{86,108}

IUI and IVF is the relatively high cost of treatment. We have demonstrated that the addition of native macaque DEF126 protein to human sperm from *del/del* donors greatly improves the ability of these sperm to penetrate hyaluronic acid gels (T. Tollner, unpublished work). This is similar to data that have been published for non-human primates—sperm lacking DEF126²⁶ are severely debilitated in cervical mucus but addition of DEF126 fully restores their ability to penetrate this visco-elastic reproductive tract medium. As such, we envision the possibility of adding recombinant DEF126 to *del/del* human sperm, thereby enabling therapeutic approaches such as vaginal or cervical artificial insemination, that are cheaper and less invasive than the currently available options, or perhaps treatment of sperm with a vaginal gel that, during intercourse, provides sperm with DEF126 as they move through the gel and into the cervix.

Advantageous properties of β -defensins

Mammalian sperm are exposed to hundreds of proteins during epididymal maturation⁷¹ and potentially many more during ejaculation.⁷² A pertinent question is why, in some species, β -defensin has become the dominant molecule for the sperm surface coat. β -defensins have two features that make them suitable to be carried by sperm into the female reproductive tract: their interactions with phospholipid membranes and their immunologic activity.

Interaction with phospholipid membranes

Defensins and other antimicrobial peptides kill microbes by binding to and disrupting their plasma membranes.

The cationic and amphipathic properties of antimicrobial peptides promote favourable interactions with negatively-charged phospholipid head groups on the outer leaflet of the plasma membrane of the target cell. The term 'carpet' is used to describe the reversible accumulation of peptides on the outer leaflet of the bilayer. Insertion of defensins into the lipid membrane of sperm—as opposed to binding to sperm proteins or carbohydrates, many of which are domain-specific—would ensure seamless coverage of all surfaces of the spermatozoa. Thus, the reason DEF126 is well-suited for uniform addition to the entire sperm surface as they pass through the epididymis, and later removal during capacitation, might lie in the biophysical properties of its β -defensin core.

From an evolutionary perspective, it might be hypothesized that the high levels of β -defensins and other antimicrobial peptides in the male duct system throughout phylogeny (Box 3) have created an opportunity for chance generation and selection of novel defensins. It is plausible that DEF126 was one such novel molecule, with a β -defensin core that promotes sperm-membrane association and a carbohydrate-rich tail that provides a uniform negative surface charge for protection and efficient sperm movement in the female tract. Indeed, some suggest that the role of defensins in the epididymis is currently evolving from antimicrobial agents of the male tract to supporters of sperm function.⁷³

Immunologic activity

The provision of antimicrobial peptides alongside male gametes seems to be a conserved phenomenon of sexual reproduction (Box 3). In some species, a clear benefit to the reproductive health of the female and conceptus has been demonstrated following delivery of male-derived antimicrobial peptides.^{74–76} In mammals, DEF126 is the only defensin described thus far that is conveyed by sperm into the female tract.^{10,15,53} While nothing is yet known about the activity of the released molecule in the female, nonglycosylated recombinant peptides of DEF126 in humans⁷⁷ and Defb22 in rats⁷⁸ have demonstrated antibacterial activity *in vitro*. In addition, nonglycosylated recombinant mouse Defb22 promotes chemotaxis of neutrophils (T. Tollner, unpublished work). Notably, around the time of ovulation there is reduced expression of endometrial β -defensins in women.⁷⁹ Perhaps DEF126 released by sperm fulfils an apparent midcycle gap in female innate defenses, a time when barriers to microbial infiltration into the upper female reproductive tract are lessened. Therefore, an attractive hypothesis is that DEF126 provides immunologic protection to both mother and embryo.

Conclusions

DEF126 (and its orthologs in other mammals) seems to fulfil several requirements of internal fertilization. Chief of these is the provision of a dense, negatively charged sperm glycocalyx. Thereby, DEF126 imparts properties to the surface of sperm that are essential for multiple sperm functions in the female reproductive tract.

The negatively charged glycans of DEFB126 facilitate efficient sperm movement through cervical mucus and cloak sperm from immune surveillance. Sperm that reach the oviduct can tether to the epithelial lining via DEFB126, enabling sperm to be stored until the time of ovulation. Conditions consistent with periovulatory oviductal fluid trigger the release of DEFB126 from sperm, an event that might be critical for releasing sperm from oviductal reservoirs and preparing sperm to interact with the egg and its vestments.

Not surprisingly, a common mutation in the *DEFB126* gene in men results in sperm with an altered glyco-calyx, inferior sperm function, and reduced fertility. Conceivably, a surrogate molecule sharing the key structural features of DEFB126 could be added to the defensin-deficient sperm of men homozygous for the *del* mutation, restoring the glyco-calyx and promoting fertility. Future studies will reveal the extent to which the

genetic mutation is responsible for idiopathic male infertility, and whether DEFB126 augments innate immunity in the female reproductive tract.

Review criteria

We searched a number of databases (PubMed, Google Scholar, ISI Web of Science, and Agricola) using the search terms “oviduct”, “cervical mucus”, “sperm transport”, “epididymis”, “sperm maturation”, “capacitation”, “sperm glyco-calyx”, “beta-defensin”, “antimicrobial peptides” and “reproductive tract immune response” for English-language papers without limits on the year of publication. We were able to access full-length versions of most papers online and accessed full-text versions of all other papers at one of two libraries at the University of California, Davis or through interlibrary loan access. In many cases, the content and reference list of research and review articles were mined for additional sources.

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