

Protamines and spermatogenesis in *Drosophila* and *Homo sapiens*

A comparative analysis

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The production of mature and motile sperm is a detailed process that utilizes many molecular players to ensure the faithful execution of spermatogenesis. In most species that have been examined, spermatogenesis begins with a single cell that undergoes dramatic transformation, culminating with the hypercompaction of DNA into the sperm head by replacing histones with protamines. Precise execution of the stages of spermatogenesis results in the production of motile sperm. While comparative analyses have been used to identify similarities and differences in spermatogenesis between species, the focus has primarily been on vertebrate spermatogenesis, particularly mammals. To understand the evolutionary basis of spermatogenetic variation, however, a more comprehensive comparison is needed. In this review, we examine spermatogenesis and the final packaging of DNA into the sperm head in the insect *Drosophila melanogaster* and compare it to spermatogenesis in *Homo sapiens*.

Introduction

Spermatogenesis is a highly orchestrated process that requires the correct interplay and timing of all molecular constituents to produce fully functional and motile sperm. Defects in spermatogenesis can impact a male's overall fitness, which encompasses the ability to both survive and reproduce successfully.¹⁻⁵ Aberrations during any stage within spermatogenesis can have profound effects on sperm quantity, motility, morphology and ability to fertilize an egg. In addition, poor packaging of chromatin within sperm nuclei can reduce the protection of DNA against chemical and physical damage, potentially leading to mutations and unfit offspring.

Spermatogenesis can be divided into three stages: pre-meiosis, meiosis and post-meiosis. During the course of the latter stages, two major transformations occur that drastically alter the cells produced from a single stem cell into mature sperm. First, primary spermatocytes developed in the pre-meiotic stage will transition during meiosis from a diploid genome to a haploid genome (reviewed in ref. 6). Second, modifications that occur

post-meiotically act to condense chromatin to fit within the sperm nuclei.^{7,8} Modifications to sperm chromatin require the use of specialized DNA-binding proteins, referred to as protamines, which are capable of achieving the level of organization and compression necessary to fit the haploid genome into the compact sperm head.^{9,10}

Much is known about protamine biochemistry, genetics, molecular structure and function in vertebrates, specifically mammals (reviewed in refs. 9 and 11). Recent work has also begun to identify the role of protamines in invertebrates, such as *Drosophila*.¹²⁻¹⁴ Although differences are apparent between *Homo sapiens* (human) spermatogenesis (reviewed in refs. 7 and 15) and *Drosophila* spermatogenesis (reviewed in ref. 16), the three main stages can be observed in both groups. Unfortunately, vertebrate and invertebrate spermatogenesis are rarely compared with each other, limiting our understanding of its evolutionary history and the different selection pressures that may exist. Here, we provide an overview of *Drosophila* spermatogenesis, with a particular emphasis on protamines and DNA packaging, and compare the process to what is observed in vertebrate spermatogenesis.

Drosophila Spermatogenesis: Cytological and Molecular Overview

Spermatogenesis in *Drosophila* has long been a model system for fertility, cell signaling, cytoskeletal/cytogenetic modifications, gene expression and evolution. The stages of spermatogenesis are readily identifiable through phase contrast microscopy, as they possess stereotypical morphology that appears to be well conserved among numerous species, and the genetic tools available in *Drosophila melanogaster* allow for relatively easy identification and characterization of the molecular underpinnings of spermatogenesis. Comparisons of spermatogenesis among species can provide some generalizations about the spermatogenic process. Spermatogenesis occurs within the testes, progressing distally from the initial stem cell division at the apical hub (Fig. 1A). Here, a group of eight to nine germline stem cells (GSCs), each surrounded by a pair of cyst progenitor cells (CPCs), physically associate with a somatic support hub, which allows for self-renewal as the GSC divides.¹⁷ The cell that maintains physical association has the Jak-STAT pathway activated through somatic secretions, which returns the cell to a GSC state, providing the

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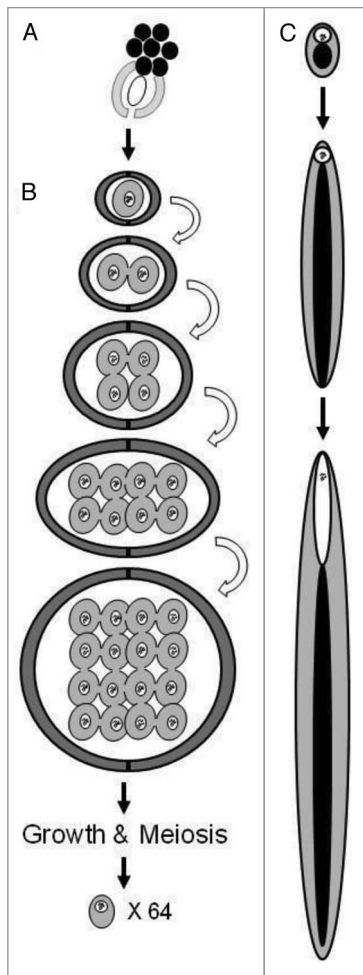


Figure 1. Spermatogenesis in *Drosophila melanogaster*. (A) Somatic hub cells (black) physically associate with GSCs (white oval) and CPCs (light gray). Both the GSCs and CPCs undergo self-renewal divisions, producing a (B) pair of daughter cyst cells (dark gray) surrounding a primary spermatogonium (gray). Black squiggles represent chromatin; note that chromatin varies in conformation depending on the stage in spermatogenesis, and this variation is not represented in the figure. The primary spermatogonium undergoes four mitotic divisions (arrows) resulting in a cyst of 16 primary spermatocyte cells. The spermatocytes will then undergo growth followed by meiosis to produce a cyst of 64 spermatids, each containing a nuclear (white circle) and mitochondrial (black circle) genome. (C) The mitochondria of each of the 64 spermatids aggregate to become nebenkern (black circle). The nucleus (white circle) maintains its size as the cell and nebenkern elongate to form the comet stage cell. As the cell elongates further, into the canoe stage cell, the nucleus begins to elongate and forms a shape that takes the appearance of a canoe with continuous extension of the former nebenkern.

template for the next round of division. The cell that is not in contact displaces from the hub and becomes the primary spermatogonium.^{17,18}

The primary spermatogonium (Fig. 1B), also known as the gonioblast, enters the first mitotic stage of spermatogenesis to produce secondary spermatogonia. Further rounds of mitosis then occur, and the number of mitotic divisions is species-specific.^{19,20} Incomplete cytokinesis occurs after each round of mitosis, causing the secondary spermatogonia to remain interconnected by

cytoplasmic bridges.^{21,22} Following meiosis, these cytoplasmic bridges may allow for the active or passive exchange of proteins that are post-meiotically transcribed,^{23,24} as seen in mammalian spermatogenesis.²⁵⁻²⁸ However, there is evidence in mice that some proteins are not shared equally (or at all) among the cells within a syncytium,²⁹ which is potentially also true in *Drosophila*. As such, this can potentially produce an imbalance of protein product availability among different spermatids.

Following the final mitotic division, a syncytium of cells called primary spermatocytes (16 in *D. melanogaster*)¹⁶ is formed.³⁰ Immature primary spermatocytes are morphologically indistinguishable from the preceding secondary spermatogonia. However, shortly after their formation, the immature primary spermatocytes begin drastic cellular and molecular changes as they enter interphase, where the cellular volume is estimated to experience a 20–25-fold increase. Upon completion of this growth phase, the primary spermatocyte will become the largest proliferating cell within the testes, and one of the largest cells in the organism.^{16,31}

A second characteristic of the primary spermatocyte is its increase in transcription levels. Primary spermatocytes produce transcripts that are used at three different time points in spermatogenesis (reviewed in refs. 32 and 33). The first group of transcripts are produced to allow for the expansive growth that takes place during the immature primary spermatocyte stage. The second group of transcripts are those that are required for meiosis, where transcription effectively ceases. The third group includes transcripts that are used post-meiotically. These transcripts are translationally repressed until after meiosis.

The beginning of meiosis is marked by the simultaneous condensation and appearance of visible bivalents. In *D. melanogaster*, this appears as three condensed aggregates, each corresponding to a bivalent pair of homologous chromosomes.^{34,35} The bivalents at this stage typically occupy a region at the nuclear periphery.^{34,36} Here, they are maintained until migration toward the metaphase plate commences during the progression of meiosis. They congregate, appearing as a single large mass in the middle of the nucleus, where they are then pulled to distinct poles during anaphase I. As previously mentioned, cytokinesis occurs incompletely, and the cell quickly enters meiosis II.

After meiosis II is complete, the cells enter the “onion stage,” which is one of the most easily observed stages in *Drosophila* spermatogenesis. During this stage, all of the mitochondria within a single secondary spermatocyte fuse into two large mitochondrial derivatives. These two large derivatives interlock, forming a massive structure called the nebenkern (Fig. 1C).³⁷ Due to the layered structure of the nebenkern, the appearance under electron microscopy looks like the layers of an onion, hence the name “onion stage.” The nebenkern becomes situated on one side of the secondary spermatocyte, while the nucleus lines up on the opposite side of the cell.³⁷ The role of this stage is not clear, but its presence throughout insect spermatogenesis indicates that it likely has a vital role.

During the final stages of spermatogenesis, the nebenkern and nucleus of the developing spermatids undergo drastic remodeling (Fig. 1C).³⁸ Initially, the nebenkern elongates down the

axoneme,¹⁶ which will become the tail, producing a shape known as the “comet” stage. During this event, the chromatin condenses at the periphery of the nucleus. Subsequently, there is nuclear elongation resulting in the “canoe” stage, then the nucleus transitions from canoe-shaped to needle-shaped, and finally individualization of the sperm cell commences.³⁹ Most of the cytoplasm is removed by individualizing machinery that traverse the bundle from the head of the sperm to the base. The last stage of sperm development within the bundles is coiling: following individualization, the entire cyst is coiled, after which the sperm are released into the testes.¹⁶ The sperm make their way to the seminal vesicle, through a currently undefined process, where they are stored until ejaculation.

While the overall morphology of *Drosophila* and human sperm are grossly similar—they both have a head and tail—they otherwise have divergent morphology resulting from differences in the spermatogenesis process.^{15,16} The sperm of *Drosophila* are long, strand-shaped structures, whose head and tail are almost indistinguishable by light microscopy; the head of the human sperm is a flattened pear shape that is easily distinguishable from the tail. During sperm elongation in *Drosophila*, each unit of the nebenkern extends along the length of the axoneme, ultimately forming the sperm tail. In humans, there is not a nebenkern, and instead the elongation process deposits individual mitochondria adjacent to the sperm head, occupying merely a third of the length of the tail.

The Role of Protamines in Spermatogenesis

During the post-meiotic stage of spermatogenesis, sperm undergo their final transformation and mature into fully functional and motile sperm. An integral process during the course of spermiogenesis is the reorganization of DNA into a highly condensed state that allows for proper packaging within the sperm head. In both vertebrates and invertebrates, the transition involves a series of steps that utilize transitional proteins that gradually replace the chromatin’s histones with protamines. In mammals, histones are removed after meiosis and are replaced by two major types of transition proteins (TP) that bind to DNA: TP1 and TP2. These transition proteins are known to condense DNA as they bind and replace histones, but do not condense the DNA to the degree seen in fully matured sperm.^{40–46} Although further investigation is needed to uncover the functional role of these transition proteins during sperm chromatin remodeling, TP1 appears to destabilize DNA to reduce the interaction between histones and DNA,^{47,48} whereas TP2 possesses two zinc fingers that may serve to condense DNA.⁴⁹ Interestingly, neither transition protein is essential for fertility in mice.^{43,50} Thus, there is either functional redundancy among the transition proteins, or additional molecular players are present during the transition period.

Less is known about the transition stage in *Drosophila*. A testes-specific protein that is present during the transition period from nucleosome-based to protamine-based chromatin³⁹ contains a high mobility group (HMG)-box motif, which has been proposed to play a role in remodeling DNA.⁵¹ Although this protein may be a strong candidate for a *Drosophila* transition protein,

its sequence is not homologous to known mammalian transition proteins;³⁹ as such, it has been named *transition protein-like*^{94D} (*tp^{P4D}*). The gene *Mst77F* also encodes a male-specific protein that has been proposed to act as either a transition protein or a protamine-like protein.⁵² This gene has been demonstrated to be critical for both male fertility and nuclear shaping in *D. melanogaster*,¹² and has high-sequence similarity to both the linker histone family histone H1/H5¹² and hHILS, which is a linker histone H1-like protein that is sperm-specific.⁵³ However, *Mst77F* differs from hHILS in that it accumulates and remains in the sperm nucleus until after fertilization has occurred.¹² *Mst77F* appears to play a role in sperm nuclei morphology, but does not affect condensation.¹⁴ Specifically, during the later stages of spermiogenesis, tubulin is weakly detectable resulting in unstable perinuclear microtubules in *Mst77F* mutants.¹⁴ This presents an interesting aspect to sperm-specific proteins, wherein *Mst77F* plays a role in nuclear shaping by affecting microtubule stability.¹⁴ The gene also appears to have a dosage-dependent effect on male fertility: a single point missense mutation within the coding region results in fully sterile males with tiny nuclei, while a mutation within the promoter region greatly reduces the amount of transcript but does not appear to affect fertility.¹²

During the final stages of sperm transformation, transition proteins are replaced by protamines, which further condense DNA to the level of compaction needed to be properly packaged within the sperm heads,^{7,8} thus increasing sperm hydrodynamics and fertilization potential. Protamines are chromatin-binding proteins that serve to condense and organize the DNA within sperm heads, and as such, they are rich in amino acid residues that are known (human) or thought to be (*Drosophila*) involved in chromatin binding and linking neighboring protamines.^{9,54} In the past three decades, research in understanding the molecular interaction between protamines and DNA have generated a proposed model for the protamine-DNA complex and the conformation of chromatin within the sperm head (Fig. 2A). The DNA-binding domain wraps around one loop of DNA, fitting into the major groove.^{9,55–58} Protamines possess a high content of arginine and cysteine residues, a trend which is seen across a wide variety of species. The arginine residues reside within the DNA-binding domain, while the cysteine residues bind neighboring protamines, thus locking the DNA strands together.^{12,56,59–63} The protamine-based chromatin then coils into a toroid, or doughnut, conformation,^{44,64} wherein the DNA lies in side-by-side arrays. Protamines have a net positive charge that, when bound to DNA, neutralizes the negative charge from the DNA phosphate backbone.^{56,58}

Extensive studies on the genes that encode protamines have mostly been performed in vertebrates, particularly in mammalian models.⁹ Human protamines (henceforth known as hProts) are often denoted as “true protamines” and their structure and amino acid composition differs greatly from *Drosophila* protamines (henceforth known as dProts), which are also referred to as protamine-like.⁶⁵ In humans and other mammals, two main families of protamines have been identified: protamine P1 (hProt1) and protamine P2 (hProt2), encoded by the genes *PRM1* and *PRM2*, respectively. hProt1 has been well characterized: the N terminus

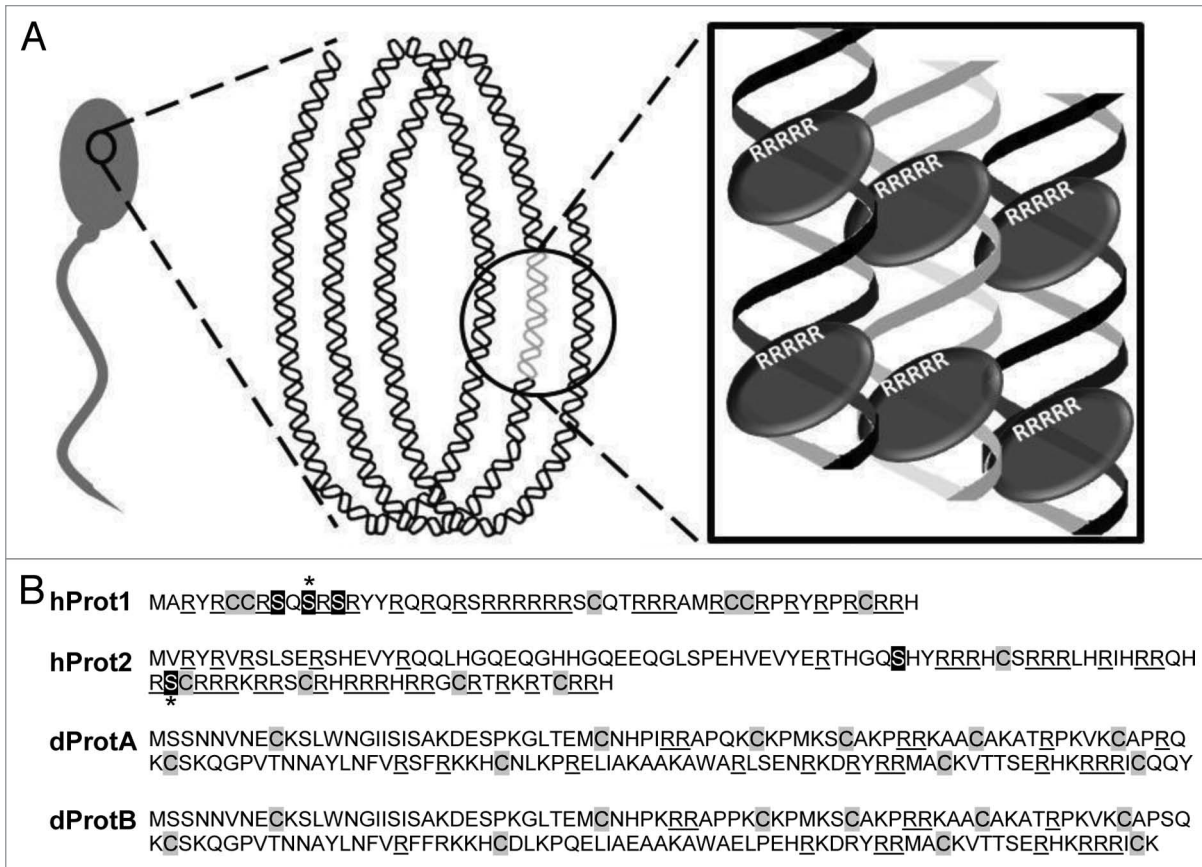


Figure 2. Proposed model for protamine-DNA complex and the conformation of DNA within a single sperm head (adapted from ref. 64). **(A)** Within the nucleus of a sperm, DNA is coiled into a doughnut shape conformation. In order to achieve this, arginine residues (white R's) of protamines (gray ovals) bind to chromatin within the major groove of the DNA. Further interaction and binding between cysteine residues (not shown) of neighboring protamines lock adjacent DNA strands into place. **(B)** Amino acid sequences of human protamines 1 and 2 (hProt1 and hProt2, respectively) and *Drosophila* protamines A and B (dProtA and dProtB, respectively). Arginine residues are underlined; cysteine residues are highlighted in gray; serines that are phosphorylated in hProt1 are highlighted in black; the serine with the greatest phosphorylation is indicated with an asterisk.⁶⁶

of hProt1 contains a high serine and threonine content, which upon phosphorylation, targets the DNA for protamine deposition (Fig. 2B).^{58,66} The binding patterns of hProt2, unlike hProt1, remain poorly understood; however, it is believed that they may be similar to hProt1.⁴⁴ The precursor of hProt1 has an initial size that is much larger than that of hProt2, but hProt1 undergoes multiple modification events until the two proteins are eventually equivalent in size.⁶⁷⁻⁶⁹ Like hProt1, hProt2 also possesses a string of arginine residues that act to bind the protamines to DNA,^{70,71} and is rich in cysteines, which are involved in the disulfide cross-linkage between neighboring protamines, either of the same or different family group.⁷²⁻⁷⁴ While hProt2 is also phosphorylated, this occurs at serine residues toward the center of the molecule rather than the N terminus, potentially indicating that hProt1 and hProt2 have different DNA binding patterns or different molecular interactions with other protamines (Fig. 2B).⁶⁶

There are two transcripts in *Drosophila* whose sequences are similar to the protamine sequences found in humans: *Mst35Ba* and *Mst35Bb*.¹² Further analysis determined that *Mst35Ba* and *Mst35Bb* encode chromatin proteins that serve to organize DNA to a level of compaction that would fit within the tiny

volume of the sperm head and, thus, these proteins are denoted as *Drosophila* protamine A (dProtA) and *Drosophila* protamine B (dProtB), respectively.¹² dProtA and dProtB are encoded by adjacent genes located on chromosome 2L and possess identical sequences for the 5'UTR, coding and 3'UTR regions, suggesting that they originated from a duplication event.¹² When both protamine genes are deleted, sperm become more susceptible to mutation when exposed to X-ray irradiation, indicating that protamines, or the increased coiling they provide, serve to protect DNA from damage.¹⁴

Structurally, hProt1 and hProt2 differ in both their size and composition (Table 1).^{12,65} hProt2 contains twice as many amino acids as hProt1, but a smaller proportion of these are arginines and cysteines. In contrast, both dProtA and dProtB have almost identical amino acid composition and size, which indicates that they are likely functionally redundant. Amino acid composition differs greatly between human protamines and *Drosophila* protamines, particularly the lysine content, which is high in dProt1 and low in hProt1. However, both the hProt1 and the dProt1 contain a relatively high content of arginine residues, which are likely involved in DNA binding, and cysteine residues, which

likely bind protamines together through disulfide bonds (Fig. 2, Table 1).^{12,65} As such, the functional similarities between hProt and dProt may be high, and their differences in amino acid length and their amino acid content could be a simple reflection of their divergent evolutionary histories.

Protamine Expression during Spermatogenesis

There are two striking differences between human and *Drosophila* protamine expression. First, male fertility in humans requires that both alleles of each protamine gene are fully functional; both genes are haploinsufficient when mutated.⁷⁵ In *Drosophila*, however, when both copies of dProtA and dProtB are simultaneously deleted, approximately 20% of sperm nuclei appear misshaped, but overall fertility is not affected. Thus, dProtA and dProtB are not essential for male fertility and may be functionally redundant with as-yet-unidentified nuclear proteins that serve to condense sperm chromatin.¹⁴ This contrasts with the previously discussed histone linker-like protein, *Mst77F*, which is essential for male sterility in a dosage-dependent manner.¹²

Second, the temporal expression of human and *Drosophila* protamine transcripts differ during spermatogenesis. In humans, both hProt1 and hProt2 are expressed post-meiotically, thus only one copy of each gene is expressed within the sperm head.^{11,76} Therefore, only one allele (from one parent) is expressed within each individual sperm derived in the offspring. However, during the course of spermatogenesis, sperm are interconnected via cytoplasmic bridges, allowing for the developing sperm to potentially share protamine transcripts.⁷⁷ What is unknown is the extent that sharing occurs between maturing sperm, and whether subtle differences in protamine expression between two sibling sperm can cause differential fertilization success. In contrast to humans, in situ hybridization in *D. melanogaster* uncovered the presence of dProtA and dProtB transcripts in primary spermatocytes, which are diploid and have yet to undergo meiosis.¹² Further research examining the allelic expression of protamines for two sibling species within the *Drosophila* genus demonstrated that both alleles for dProtB are present in each sperm cell.¹³ Therefore, in *Drosophila*, both parents contribute equally to the protamines found within a single sperm head.

The evolutionary forces acting on the temporal expression patterns of protamines, leading to differences between species, have yet to be determined. Variation in sperm functionality would have the greatest impact in species that experience sperm competition, either between individuals or within the ejaculate

Table 1. Comparison of *Drosophila* and human protamines

Protamine	Total # aa	% Cysteine	% Lysine	% Arginine
hProt1	51	11.8	0	47.1
hProt2	102	4.9	2.0	32.4
dProtA	146	6.8	14.4	12.3
dProtB	144	6.9	15.3	10.4

The total number of amino acids (aa) and percent of major amino acids are listed. (Information adapted from refs. 12 and 65).

of a single male.⁷⁸ This raises the question of whether there is a selective advantage of either haploid or diploid expression of protamines, as well as the number of species that use haploid vs. diploid protamine expression. When only one protamine allele is expressed, phenotypically different sperm could potentially be produced within one individual,^{11,76} which may increase a male's fitness if this variation translates into a greater overall fertilization success. When both protamine alleles are expressed, however, there is functional duplication of the protamines, making it less likely that a defect in either gene would impact fertility; this could also increase a male's overall fertilization success.⁷⁹⁻⁸² Thus, the different allelic expression patterns could have resulted from different selective pressures in vertebrates vs. invertebrates. These selective pressures could have been historically influential but are no longer present, could be ongoing or could even be opposite in contemporary times to what they were when these divergent expression patterns initially arose. Thus, it will be difficult to assess the evolutionary basis of protamine expression patterns. Further research examining spermatogenesis in a variety of species will allow for a more thorough comparison between the stages of spermatogenesis and the packaging of DNA into the sperm head. This will enhance our understanding of how these processes have evolved, and how variations in these processes affect a male's overall fitness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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