COMP-1 REGULATES MALE AND HERMAPHRODITE BIAS OF SPERM SELECTION IN *CAENORHABDITIS ELEGANS*

by

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ABSTRACT

Sperm competition is a pervasive mode of sexual selection across the phyla that have crucial implications in microevolutionary and macroevolutionary processes. Furthermore, females can bias the selection of sperm from one male over another in a process known as cryptic female choice. While sperm competition has been the subject of intense experimental research, very little is known about the molecular pathways and cellular mechanisms that regulate these processes. Insight into the molecular pathways regulating sperm competition and cryptic female choice are likely to have a high impact in the field of evolutionary biology since so little is currently known.

The nematode *C. elegans* offers many advantages for the study of sperm competition. Male sperm exhibit a robust competitive advantage over hermaphrodite self sperm, resulting in the almost complete preferential use of male sperm. We have identified a gene, *comp-1,* that regulates several sperm behaviors leading to reduced male precedence when competing with both self sperm and other male sperm. Critically, mutant males and hermaphrodites have normal fertility, suggesting that mutants produce and transfer as many functional sperm competent for motility, sensation of guidance cues, and fertilization as do wild type. We have shown that the *comp-1* sperm behavioral defects are specific to competitive contexts as *comp-1* sperm are functionally normal when wild type

sperm are absent. Surprisingly, wild type sperm appears to inhibit *comp-1* sperm by altering the chemical environment of the hermaphrodite, most likely to involve prostaglandin signaling. Intercellular communication between the reproductive tract and sperm is fundamental for sperm migration, and prostaglandins have been previously identified as important for this process. However, this is the first time that prostaglandins have been implicated as necessary for sperm competition. Furthermore, prostaglandins are a novel mechanism of cryptic female choice and add to the field where very little is known about how females influence sperm competition. Results from our studies of *comp-1* will provide insight into the molecular pathways necessary for sperm competition and cryptic female choice, which will ultimately broaden our knowledge of the mechanisms of evolutionary change.

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CHAPTER 1

INTRODUCTION

Evolutionary Advantages of Polyandry

Bateman's principle postulates that for most species, a male's reproductive success is only limited by how many females he can successfully inseminate, whereas the number of partners a female has should not have any impact on her fecundity (Bateman 1948). This principle, along with several explanations of why it might benefit females to have fewer partners, has supported the perception that females regularly practice monogamy in many species. In general, mating is a costly endeavor that requires high levels of energy that the female could use to procure other crucial resources. Moreover, there are several risks to both invertebrate and vertebrate females that increase with the more mates she takes, such as disease transmission, physical harm from the males, infanticide, and reduced life expectancy (Daly 1978; Chapman et al. 1995). It seems that mating only to produce offspring would be in the female's best interest. Yet, female promiscuity is regularly practiced in many taxa, including, insects, birds, fish, reptiles, and mammals (Birkhead and Moller 1998). As a result, the sperm from more than one male is often residing in the female reproductive tract, forcing sperm from different ejaculates to compete with each

other to successfully fertilize a single set of eggs. While sperm competition has largely been viewed as an extension of the contest between males for mates, the role of the female in selecting more partners and influencing the pattern of paternity is becoming more prominent.

The premise of sexual selection in most species is that an individual's fitness depends on their ability to spread their genes to future generations. For males, it is in their best interests to mate with as many females as they can, thereby increasing their chances of successful fertilizations (Birkhead and Moller 1998). On the other hand, females are limited by the number of viable eggs they produce or by the number of young they can nurture at one time (Trivers 1972). Once the female's maximum capacity for fertility and childcare has been met, it is proposed that the costs of mating would prohibit the female from mating with more males. The advantages of polyandry are much less obvious, but several conspicuous behaviors can explain the asset of more male partners. For example, males can provide resources to prospective mates, increasing the female's nutrient intake in the form of nuptial gifts such has been observed in insects (Gwynne 1984), a benefit to both herself and her young. However, even without material benefits from males of mammals, birds, and insects, competitive ejaculates can increase the female's fitness by increasing offspring survival and producing fitter offspring (reviewed in (Parker and Birkhead 2013)). A simple nongenetic hypothesis explaining the motivation for polyandry is that it ensures that females are reaching their maximum fecundity by guarding against low sperm stores or poor sperm quality (Sakaluk and Cade 1980; Walker 1980). A

female could replenish her sperm stores or hedge against infertility by mating with one or two partners. However, these hypotheses do not adequately explain the benefit of multiple partners observed in many species. A more profound explanation would entail affecting the organism's fitness at the genetic level, thereby falling within the definition of sexual selection.

Offspring quality

Attempts to explain polyandry have historically been male centric. A male with high-quality sperm outcompetes other male ejaculates to fertilize the oocyte. The good-sperm hypothesis suggests that the male's sperm quality, and thus successful competitive ability, correlates well with the quality of their overall genetics (Yasui 1997). Thus, a male with high-quality sperm will contribute good genetic material, leading to higher quality offspring and thereby indirectly benefiting the female. Several field studies have reported correlations between female adders with higher number of partners having fewer stillborn young (Madsen et al. 1992), consistent with the good-sperm hypothesis. Although the good-sperm hypothesis provides a plausible explanation for polyandry, later studies found that the increase in hatching was due to genetic compatibility between the male and female. Therefore, the responsibility of embryo viability is one that is shared between the male and female (Tregenza and Wedell 1998; Newcomer et al. 1999; Simmons 2001a). These data alone produce a conundrum in that although polyandry overall is favorable to the female and the hatching success of her future offspring, sperm competition itself would remain

as a process that provides benefits only to the male. However, another study demonstrated that sperm competition does result in offspring with faster development, a quality likely to be advantageous to the female's offspring. In light of this finding, polyandry likely evolved to produce fitter offspring, at least in early development, via sperm competition as observed in the yellow dung fly (Hosken et al. 2003). Another reason why females may want to mate with multiple males is the genetic diversity hypothesis proposed by evidence using a cricket mating system, which posits that females hedge against future environmental uncertainties by using sperm with differing genetic backgrounds (Yasui 1997, 2001).

Genetic compatibility

If increased hatching success is due to enhanced genetic compatibility between the male and female, there must be a source of genetic incompatibility. Generally, this source is the combination of the parental haplotypes producing offspring of inferior quality, which can be due to the additive effects of several loci or the nonadditive effects of one or two deleterious loci. It is widely accepted that the level of the individual's heterozygosity directly affects their fitness (Brown 1997; Tregenza and Wedell 2000), either as a result of inbreeding depression (Charlesworth and Charlesworth 1987; Keller and Waller 2002) or to increased homozygosity advancing the expression of deleterious recessive mutations or selfish genetic elements (Stockley et al. 1993; Pusey and Wolf 1996; Zeh and Zeh 1997). As proof of principle, female crickets actively select sperm from

nonsibling males when given the choice to avoid inbreeding (Tregenza and Wedell 2002).

One key functional loci whose loss of variation is particularly detrimental to offspring is the major histocompatibility complex (MHC). MHC genes encode antigen presenting molecules necessary for the organism's acquired immune response (Janeway et al. 1999). Loss of variation in these genes can compromise the individual's ability to fight off disease (Doherty and Zinkernagel 1975; Penn et al. 2002). Through cryptic female choice, wherein females favor sperm from one male over another, sperm from MHC-dissimilar partners are more likely to reach the eggs within the reproductive tract of the red jungle fowl females than MHC-similar males (Lovlie et al. 2013). These studies provide the first evidence that polyandry functions to improve the genetic compatibility of the female's and male's genetic contributions to the advantage of their offspring.

Female fitness

Female polyandry in internally fertilizing species ensures that sperm from different ejaculates overlap, which forces sperm from different partners to compete. Although sperm competition largely benefits the male's fitness, there is some evidence that sperm competition bolsters the female's fitness by contributing to the early development of offspring. However, these studies are limited, leaving sperm competition as an extraneous event to the female's reproductive interests. One could argue, if the attributes affecting sperm's fertilization success are heritable, then the female's fitness indirectly benefits by

producing sons that will more likely have ejaculates with superior sperm competitors (Keller and Reeve 1995). Experiments on invertebrates have concluded that populations maintained under polygamous conditions have increased testes size and sperm number as compared to their monogamous counterparts. The most well-described mechanism to increase a male's fertilization success is to increase sperm number and has been observed as a key contributor to sperm competition in many species including mammals, insects, and roundworms (Hosken and Ward 2001; Pitnick et al. 2001; Simmons and Garcia-Gonzalez 2008). In *C. elegans*, after several generations of intense sperm competition, males evolve larger sperm (Murray et al. 2011), a characteristic known to contribute to increased sperm competition in this particular species and in others (LaMunyon and Ward 1998b). The finding that polyandry contributes to male fertilization success is not limited to invertebrates, as polygamous house mice also display greater sperm numbers and increased swimming performance and therefore greater paternity bias over monogamous lines, even after a relatively small period of time of twelve generations (Firman and Simmons 2010; Firman 2011; Firman and Simmons 2011). While these findings may directly benefit the males, females gain greater fitness through producing sons with potential higher reproductive success.

Evolutionary Consequences of Sperm Competition

At one time, sexual selection was thought to solely rely on precopulatory mechanisms, such as attracting mates through vibrant plumage. However, the

prevalence of female promiscuity and its important biological outcomes is now widely known. A female with more than one male's ejaculate in her reproductive tract means that the male successor can be chosen up to the point of fertilization. In a seminal paper (Parker 1970), sperm competition was defined as a postcopulatory mode of sexual selection. Sperm competition is a potent mediator of sexual selection, the evolutionary process of increasing the gene frequency of traits that confer a reproductive advantage. As a likely result of sperm competition, the diversity of sperm morphology is unparalleled by any other tissue type. Sperm come in in all shapes and sizes and almost every component of sperm has been subjected to some form of modification. Of the four main components conserved in most sperm among different organisms: the acrosome, mitochondria, nucleus, and flagellum, there are examples of sperm lacking each one of them (Jamieson et al. 1999). Sperm is not the only reproductive tissue that has undergone extensive remodeling. Female reproductive tracts can be quite anatomically diverse. Perhaps one the most extreme examples can be observed in the Muscovy ducks. In order to avoid fertilization by unwanted male mates, females have evolved a corkscrew reproductive tract that twists in the opposite direction of the male's penis. Moreover, the vagina has several "dead ends" into which the female can redirect the male's penis if she deems him an inferior partner. This evolutionary adaption has been so successful that only 3% of copulations result in fertilization (Brennan et al. 2007; Brennan et al. 2010). Similar to precopulatory mechanisms, postcopulatory sexual selection is thought to drive the rapid diversification of reproductive characteristics in most taxa

(Simmons 2001a). Like the Muscovy ducks' intriguing genitalia, many species display elaborate forms of reproductive morphology and anatomy as a result of sexual selection.

Evolution of reproductive proteins

Given the complexity and diversity of sperm and reproductive tract morphologies and anatomy, it is reasonable to hypothesize that reproductive proteins might be evolving at a faster rate than proteins of other tissues. Typically, reproductive proteins are those involved with postcopulatory processes such as sperm usage, sperm storage, intercellular communication between sperm and egg, and fertilization. By comparing gene sequences between closely related species of *Drosophila*, studies have shown that genes necessary for sexual reproduction are diverging more quickly than their nonreproductive counterparts and on average have a higher amount of amino acid substitutions (Vacquier 1998; Singh and Kulathinal 2000). In the case of *Drosophila melanogaster*, the rate of divergence is occurring at twice the rate of other genes (Civetta and Singh 1995). In fact, reproductive proteins are one of the larger subset of genes that are under positive selection for vertebrates and invertebrates alike (Swanson et al. 2001a; Swanson et al. 2001b; Torgerson et al. 2002; Swanson et al. 2003), suggesting that their evolutionary change is conferring a benefit to the organism. One example of a specific group of proteins that are evolving at a rapid pace is the accessory gland proteins (Acp). In male *Drosophila,* species Acps regulate sperm storage and use, and oviposition (Ram

and Wolfner 2007). In mammals, the zona pellucida glycoprotein 3 (ZP3), necessary for the proper formation of the egg coat and thought to be necessary for sperm binding (Wassarman et al. 1999; Swanson et al. 2001b), has undergone adaptive selection exactly at the domain necessary for egg-sperm binding (Berlin and Smith 2005). The driving force for this rapid selection is thought to be due to internal selection via gamete selection by both sperm competition and cryptic female choice. Additionally, the interplay between genders is thought to contribute to the coevolution of the sexes and to be a major promoter to reproductive protein evolution (Swanson and Vacquier 2002; Clark et al. 2006b). The functional consequence of rapid reproductive protein divergence provides a mechanism sufficient to reproductively isolate populations or closely related species. As in the case of ZP3, one could imagine the functional consequences of a single amino acid change in the domain necessary for sperm-egg binding could have drastic effects on the successful fertilization between a male and female, thus providing a mode to successfully prohibit the reproductive success of a male and female from two populations.

Speciation

Sperm competition and cryptic female choice is thought to drive the rapid diversification of reproductive traits, leading to the hypothesis that in isolated populations, it can also drive the formation and maintenance of species boundaries (Markow 1997; Parker and Partridge 1998; Eady 2001; Kraaijeveld et al. 2011). Several characteristics broadly influence male fertility and sperm

competition, including male genitalia, seminal fluid composition, sperm traits, and female reproductive tract morphology and secretory chemistry (Eberhard 1985; Snook 2005; Poiani 2006; Pitnick et al. 2009a; Pitnick et al. 2009b; Leonard and Cordoba-Aguilar 2010). The complexity of ejaculate-female interactions, along with the seeming coevolution of these interactions and their rapid evolution (Ram and Wolfner 2007; Pitnick et al. 2009b), suggest a potential role of these interactions in species isolation. However, associating the microevolutionary processes of changes in reproductive chemistry or morphology with the broader macroevolutionary concept of speciation has been difficult to determine. Studies in *C. elegans* provide some of the best evidence of functional diversification as a direct result of sperm competition, wherein sperm increase in size due to intense sperm competition (LaMunyon and Ward 1998b). While this confirms that sperm competition can cause phenotypic changes in sperm morphology, differences in morphology are only correlated with the macroevolutionary process of speciation. In order to achieve reproductive isolation, ejaculates of another population or species would need to be prevented from fertilizing the oocytes at one of the key reproductive events. Such events would either be in the form of blocking sperm from being transferred, mislocalizing sperm within or completely ejecting sperm from the reproductive tract, or inhibiting fertilization. In an elegant study measuring sperm performance between the recently diverged *D. mauritiana* and *D. simulans* (Manier et al. 2013), it was found that all of the reproductive events contributed to conspecific sperm precedence between the two species. Additionally, *Caenorhabditis* nematodes' interspecies mating caused sperm to

inflict male-induced harm, leading to sterility of the hermaphrodite (Ting et al. 2014). These results support a hypothesis in which sperm competition and cryptic female choice can cause the rapid evolution of the ejaculate and female reproductive tract so that incompatibilities restrict gene flow between populations or species.

Mechanisms of Sperm Competition

Since Geoff Parker's seminal paper in 1970 (Parker 1970), studies of sperm competition have revealed many behaviors and traits contributing to one male's fertilization success over another (reviewed in (Wigby and Chapman 2004b)). Of particular importance in understanding these traits is solving the mechanisms by which sperm achieve a competitive advantage. One of the first proposed models of how sperm attain fertilization success was the "fair raffle system," in which individual sperm have no competitive superiority over another and each male has an equal chance to fertilize the egg. It was hypothesized sperm competition was achieved by a numbers game with the probability of each male in fertilizing an egg was in relation to the proportion of sperm they transferred as compared to the total sperm present (Parker 1990; Parker et al. 1990). More simply, the male has a greater chance of fertilization success when more sperm are successfully transferred. An increase in testes size, an indicator of increased sperm production, is one of the most described evolutionary responses to sperm competition across species (Birkhead and Moller 1998; Simmons 2001a). It is widely expected that in many taxa, sperm production

increases with the intensity of sperm competition (Parker and Pizzari 2010). However, it has since been demonstrated that precedence was not always determined by a numbers game and that the mechanisms determining sperm competition were much more complex. In many species, the order of mating can determine paternity, such as in insects and birds where generally the second male to mate will fertilize the majority of the females eggs, known as last male sperm precedence (Parker 1984; Parker et al. 2010). The opposite can occur wherein preference is given to the first male (Jones et al. 2002). Male sperm compete both offensively and defensively, meaning that previously stored sperm must resist removal or inactivation while incoming sperm must displace sperm already present. Sperm competition can also be determined by some underlying compatibility between the sperm and the female that appears to defy the rules of who came first or last. The order of mating or the seemingly randomness determining male precedence highlights the variety of mechanisms sperm employ to gain precedence, and suggest that there are intrinsic differences between ejaculates that give individual sperm a competitive advantage. Historically, sperm competition measurements typically have been indirect due to the difficulties of discriminating sperm from different ejaculates and observing sperm behaviors directly within the selective environment. Particularly elusive are the molecular pathways involved in regulating sperm competition. However, several advances have been made and we are beginning to understand the cellular mechanisms and elucidate the genes necessary for their function in sperm competition.

Sperm size and velocity

In response to sperm competition being ruled by a fair raffle, most male animals produce many, tiny sperm. However, some species produce giant sperm, such as *Drosophila bifurca* where sperm length is 20X the size of the total body length of the organism producing such sperm (Pitnick et al. 1995). Larger sperm are costly to produce both in terms of the sperm themselves and the size of the testes that hold them, and in a direct tradeoff model, the male would not likely be able to manufacture as many sperm (Immler et al. 2011). In *C. elegans* males, strains with larger sperm produce them at a slower rate (LaMunyon and Ward 1998b; Murray et al. 2011). Such a large tradeoff suggests that the larger sperm offer a substantially significant advantage. In moths, sperm length coevolved as a response to the female's elongating spermathecal duct, suggesting that the longer sperm navigated the longer ducts more efficiently (Morrow and Gage 2000). Additionally, it is thought that sperm length determines favorable positioning within the reproductive tract of *D. melanogaster* females (Miller and Pitnick 2002). Other explanations for larger sperm include that they may resist displacement by other sperm or block access to the site of fertilization (Wigby and Chapman 2004a), two mechanisms which would likely confer a competitive advantage.

Some of the strongest experimental evidence for sperm size conferring an advantage comes from the hermaphroditic species, *C. elegans*. *C. elegans* is a male-hermaphrodite species in which hermaphrodites produce and use their stores of self sperm, but can be inseminated by males. After mating, male sperm

outcompete self sperm, resulting in the nearly absolute preferential use of male sperm (Ward and Carrel 1979; LaMunyon and Ward 1995). In a poorly understood process, male sperm displace self sperm from the spermatheca, the site of fertilization in *C. elegans*. While the form and function of male and hermaphrodite sperm are almost identical, the size of male sperm is significantly larger than that of hermaphrodite sperm, leading many to hypothesize that size contributes to the male's advantage (Nelson et al. 1982; LaMunyon and Ward 1998a). Supporting the hypothesis that size confers an advantage, AB1 is a *C. elegans* strain that produces larger sperm and when competed against other male strains with significantly smaller sperm, paternity is biased towards the AB1 male (LaMunyon and Ward 1998b; Murray et al. 2011). Larger sperm size is thought to be more competitive due to the correlation of faster crawling speeds with larger sperm size *in vitro*. Although it is unclear if size is a selective mechanism in *C. elegans*, there is growing evidence in other organisms that supports longer or larger sperm size with faster sperm velocity (Simmons and Fitzpatrick 2012) with the idea that larger sperm reach the ova more quickly than their rivals (Gomendio and Roldan 1991). Again, there is a correlation between species of different taxa with intense sperm competition and larger and faster sperm (Gomendio and Roldan 2008; Fitzpatrick et al. 2009; Kleven et al. 2009). Even within the ejaculate of one male, there is a higher correspondence between longer sperm length and velocity, suggesting that there may be an even stronger case for sperm size affecting velocity when accounting for within male variance in other species (Fitzpatrick and Baer 2011).

Sperm cooperation

Sperm cooperation between individual sperm of a given ejaculate in which one or more motile cells aggregate to improve velocity is one of the more exciting but less understood strategies involved in regulating sperm competition (Fisher and Hoekstra 2010; Higginson and Pitnick 2010; Fisher et al. 2014). In some cases, physical contact is not even necessary as a concentration of sperm can collectively influence each other through hydrodynamic interactions (Yang et al. 2008). In the Dytiscid water beetle, sperm heads attach to each other and use the power of the their two tails combined to propel them towards the egg (Mackie and Walker 1974). In yet another example, the sperm of the Gyrinid beetles attach themselves through a rod-like structure that facilitates in coordinating movement to the sperm storage organs (Mackie and Walker 1974). Aggregates of hundreds of sperm have also been found to improve overall motility, such as in some fish where larger groups of sperm move faster towards the spermatheca than smaller groups (Hayashi 1998). In mammals, sperm of the Norway rat have a specialized sperm structure in the shape of a hook that functions to form larger, and faster, groups of sperm (Immler et al. 2007). Presumably, if aggregation operated as a mechanism of sperm competition, sperm from the same ejaculate would have a form of self recognition. However, there is an absence of evidence that these aggregates are limited to the ejaculate of one male and further experiments will be necessary to determine if the competitive advantage of these aggregations are specific to sperm of one ejaculate.

Seminal fluid

Much of the research on ejaculates has focused on the sperm, neglecting the role of seminal fluid in sperm competition. Seminal fluid proteins (Sfps) in *Drosophila* play an important role in male fertility by affecting sperm motility, capacitation, storage, fertilization capacity, viability, and female responses (Prout and Clark 2000; Poiani 2006; Wong et al. 2008; Wigby et al. 2009a; LaFlamme et al. 2012). Seminal vesicle proteins are also known to be crucial for the formation of a mating plug in mammals and insects, a temporary structure thought to prevent remating by other males and a potential mechanism of sperm competition (Simmons 2001b; Ramm et al. 2005). Given the diverse role of Sfps in sperm functions, they are expected to play a critical role in sperm competition. Sfps compose a substantial amount of the ejaculate and since they are a limited resource, it benefits the male to strategically allocate them. Interestingly, in *D. melanogaster* males, more Sfps are transferred in response to the potential level of sperm competition and males that can transfer more Sfps have a significant competitive advantage (Wigby et al. 2009b). Furthermore, male insects can modulate levels of certain Sfp proteins to change the ejaculate composition, and it is thought that modulation of seminal fluid is responsible for the increase in sperm viability when males perceive higher levels of competition (Simmons et al. 2007; Sirot et al. 2011). In the case of the domestic chicken, dominant males differentially adjust their seminal fluid allocations, resulting in increased sperm velocity, when mating to more attractive females (Cornwallis and Birkhead 2007).

In social insects, ants and bees, seminal fluid of polyandrous groups has

been implicated in improving the survival of sperm from one male while negatively affecting sperm from other males (Fry and Wilkinson 2004; den Boer et al. 2010). Interestingly, secretions from the female's spermatheca can counter this effect through cryptic female choice (den Boer et al. 2010). In the external fertilizing fish species, the grass goby, males assume one of two roles: the territorial male whose sperm normally does not compete with other males versus the sneaker males who almost always parasitize spawning nests (Scaggiante et al. 1999). Sperm performance does not differ between the two males in their own seminal fluid, however; the velocity of sneaker sperm improves in the seminal fluid of the territorial male, while the territorial sperm has reduced performance in the seminal fluid of the sneaker male (Locatello et al. 2013). These findings support the role of seminal fluid as a detriment to rival male sperm.

A major component of seminal fluid is the male accessory gland proteins (Acps), a diverse mixture of anything from prohormones to glycoproteins, that were first discovered to cause increased egg production and decreased receptivity to future mates in *D. melangastor* females (Manning 1962; Merle 1968). To date, 133 different *Drosophila* proteins have been identified that are transferred along with sperm (Findlay et al. 2008), representing the importance of seminal fluid. In particular, sex peptide (SP) *Acp70A* was identified and found to reduce the females likelihood to remate (Chen et al. 1988), a clever behavioral mechanism employed to avoid direct sperm competition. Another Acp, *Acp34DE*, is directly involved in mediating the outcome of sperm competition by affecting sperm storage in the female (Neubaum and Wolfner 1999; Tram and Wolfner

1999; Chapman et al. 2000). *D. melanogaster* has a typical insect paternity bias that favors the second male to mate with the female, which is thought to occur by displacement of the first male's sperm (Clark et al. 1995; Gilchrist and Partridge 1995). Interestingly, *Acp36DE* itself is not displacing sperm since transfer of *Acp36DE* seminal fluid without sperm did not affect the progeny count of the first male (Chapman et al. 2000), indicating that seminal fluid is promoting sperm behaviors related to displacing other sperm. *Acp29AB*, a predicted lectin, is another Acp that localizes to the female's sperm storage organs and is necessary for maintaining sperm positioning within the sperm storage organs, providing a sperm competition advantage (Wong et al. 2008). Acps mediate sperm storage by inducing morphological changes in the female's reproductive tract, which may include induction of muscle contractions that assist sperm motility towards the spermatheca and seminal receptacle (Heifetz and Wolfner 2004; Adams and Wolfner 2007). Other Acps, again predicted lectins, involved in sperm storage are necessary for the efficient release of sperm from the seminal receptacle (Ravi Ram et al. 2005; Avila et al. 2010). These, and other Acps, highlight the potential cellular role of sperm adhesion in storage as a mechanism of sperm competition. Acps divergence is greater in *Drosophila* species with strong sperm competition (Haerty et al. 2007; Wagstaff and Begun 2007), thus suggesting that more Acps are expected to play a role in sperm competition that is not yet appreciated by researchers.

Sperm-egg interactions

Postcopulatory selection can occur at three key events: insemination, sperm storage, and fertilization. Although there is no direct evidence of sperm being preferentially selected at fertilization, proteins for sperm-egg interactions are rapidly diverging and under positive selection (Swanson 2003; Clark et al. 2006a; Vicens et al. 2014), suggesting that sexual selection may be influencing sperm-egg interactions. Potential candidate genes necessary for fertilization that may be involved in sperm competition are the *Adam,* A Disintegrin and Metalloprotease*,* genes in mouse. There are at least 35 ADAMs in mammals, approximately half of which are expressed in the testes. The testes-specific ADAMs have a higher divergence than somatic-tissue ADAMs and only sperm surface proteins display positive selection at the adhesion domain necessary for sperm-egg interactions (Civetta 2003; Glassey and Civetta 2004), suggesting that these proteins have evolved under sexual selection (Finn and Civetta 2010). An alternative explanation for the high divergence and positive selection may be the coevolutionary arms race between the sperm quickly gaining entry and the female preventing polyspermy, the entry of more than one sperm into the oocyte. While ADAMs are necessary for normal fertilization, an exciting possibility is that they may also be involved in sperm competition based on preliminary evidence.

Cryptic Female Choice

Often times, it is thought that the female's main role in sexual selection is choosing a mate. Males have evolved a variety of elaborate secondary sexual

characteristics that serve no purpose in copulation, but have been selected for based on the advantage they confer to the male during courtship. An extreme example is the male peacock: the large bright feathers increase their level of predation, but are effective in attracting the opposite sex (Petrie et al. 1991). For the female, however, this limited role in mate choice can pose several problems. In many species, the frequency in which males force themselves on females potentially eliminates the female's preferred mate choice. Another issue is that females may potentially select males based on physical characteristics, using external traits as a proxy to judge the male's genetic quality as demonstrated in cricket species (Lailvaux et al. 2010). It is quite possible that a male may have all the better qualities that a female may desire, yet be too closely related to produce superior offspring. Research has shown that females have a further degree of control by biasing the postcopulatory outcome of sperm competition (Eberhard 1996). Female cryptic choice, the female manipulation of sperm selection, can circumvent the male's control for fertilization success. It also provides yet another avenue in determining the quality of the male's genetic contribution. The genetic basis for cryptic female choice is not known although several candidate genes in *D. melanogaster* have been identified that may contribute to sperm selection (Giardina et al. 2011). Unraveling the molecular basis of cryptic female choice will be challenging since it is difficult to resolve if sperm selection is a female-mediated process or due to sperm competition.

Female reproductive tract morphology

Sperm competition between males typically occurs within the female reproductive tract and it is thought that the female's anatomy plays an active role in this process. The variety of complex female reproductive morphologies led many to hypothesize that it was capable of a discriminatory role in sperm selection (Walker 1980; Linley and Simmons 1981). One such example is the female dungfly, who has a complicated reproductive morphology including multiple spermathecae in which she can separately store sperm from different males (Otronen et al. 1997; Ward 1998). Sperm must navigate these long, convoluted ducts before reaching the ova (Eberhard 1996). *D. melanogaster* is another promiscuous female species that stores sperm from multiple males for periods up to two weeks in either the seminal receptacle or spermathecae (Gilbert 1981; Marks et al. 1988). In an experiment using mutant females, researchers found that females with three instead of two spermathecae changed the pattern of sperm use over time, indicating that female morphology does bias sperm fertilization success (Bangham et al. 2003). Additionally, females can manipulate paternity by dumping stored sperm after mating with males, a process that does not require seminal fluid (Snook and Hosken 2004; Manier et al. 2010). In *D. simulans*, the female can switch between using the sperm from the seminal receptacle or the spermatheca depending on the quality of the sperm (Lupold et al. 2013). As techniques are developed to observe sperm behaviors within the reproductive tract, we will undoubtedly see more ways in which the female morphology influences sperm competition.

Chemical environment

Whether through choice or force, female chickens are one example of species that frequently practice polyandry. Commonly, the female prefers mating with dominant males, yet they can not avoid copulation with subdominant males. However, they can regain control over paternity through cryptic female choice. Researchers studied the red jungle fowl, the wild ancestor of the domestic chicken, and found that mating with different males produce offspring that are more resistant to disease. Females maximize the genetic quality of their offspring by biasing fertilization in favor of male sperm that are more genetically different from them. Specifically, they favor male sperm that can increase the diversity of MHC (Lovlie et al. 2013). Although the mechanism occurs internally in the female's reproductive tract, it is not known how the female influences sperm selection. In fact, very little is known about how females regulate their reproductive tract environment to influence the outcome of sperm competition.

Some of the earliest evidence of females regulating sperm behaviors comes from externally fertilizing fish. Along with the release of her eggs, the females discharge ovarian fluid that creates a chemical microenvironment surrounding the eggs and affects the swimming velocity of male sperm (Urbahc et al. 2005; Rosengrave et al. 2009). Modulating swimming velocity is a classic mechanism of sperm competition, and in fish it is the prime determinant for fertilization success (Gage et al. 2004). Moreover, the ovarian fluid from different females differentially affect sperm velocity, trajectory, and longevity from different males, suggesting that the variation of ovarian fluid composition could be a form

of cryptic female choice selecting male sperm based on their genotype (Rosengrave et al. 2008). The internally fertilizing fish, the guppy, is known to have polyandrous females unable to distinguish closely related males (Guervara-Fiore et al. 2010). However, the paternity in natural populations shows a tendency towards genetically unrelated males (Johnson et al. 2010). Using artificial insemination to control sperm numbers and reduce any copulatory behaviors on sperm selection, researchers found that unrelated males were more successful at siring offspring when competing against a related male even when brood sizes of both males were similar. Considering that ovarian fluid has an affect on externally fertilizing fish species, the researchers went on to show that ovarian fluid promotes increased swimming velocity of the unrelated versus related male (Gasparini and Pilastro 2011). Female frogs also employ a similar mechanisms by releasing egg jelly that acts as a chemoattractant and regulates the onset of sperm motility and swimming velocity (Simmons et al. 2009). Overall, these data support a hypothesis that female organisms are influencing sperm competition in favor of genetically unrelated males through chemical manipulation of their ovarian fluid that ultimately regulates sperm behaviors. As to what might be the determinant in ovarian fluid promoting these behaviors is still a mystery, although pH has been implicated in enhancing sperm motility in rainbow trout (Wojczak et al. 2007). In another study, conspecific ovarian fluid was shown to act as a chemoattractant that increased motility and straightened out the sperm's trajectory. Surprising, not only did conspecific ovarian fluid attract more sperm than the heterospecific ovarian fluid, heterospecific ovarian fluid had

the same poor chemoattractant properties as water (Yeates et al. 2013). Although these studies shed light on the chemical mechanism of cryptic female choice in regulating male sperm swimming behaviors through chemoattration, nothing is known about the proteins necessary to regulate the chemical composition of ovarian fluid.

Coevolution of the Sexes

Often times, postcopulatory selection is not a function of female bias or sperm competition, but rather a result of a male-female interaction via coevolution of the two sexes. This interaction can be seen in diving beetles; the reproductive tract has undergone extensive changes in shape and size along with corresponding divergence of sperm morphology (Higginson et al. 2012). One of the best examples of the concept of reproductive coevolution deciding the outcome of sperm selection is sperm length and the length of the female seminal receptacle in *D. melanogaster.* Male fertilization success is not determined by the overall length of sperm but performance is constrained by an optimal sperm length given the length of the seminal receptacle (Miller and Pitnick 2002). Another interesting intercommunication process between the two sexes involves sex peptide, a component of seminal fluid, as a master transcriptional regulator causing the induction of genes in the female related to development, early embryogenesis, and behavior (Gioti et al. 2012). However, some of the strategies developed by males in response to male-female interactions are antagonistic to the female, leading to sexual conflict. In *Drosophila,* the seminal fluid increases

the rate of female ovulation, but perhaps at the cost of the female's longevity and overall lifetime reproductive success (Chapman et al. 1995; Pizzari and Snook 2003). As such, evolution of reproductive proteins is a complex and sometimes intertwined process driven by sexual selection with inputs by the male, female, or male-female interaction, or as a result of a sexual conflict arms race where the females and males are constantly outmaneuvering the opposite sex. Future studies will necessitate careful examination of the contributions of each sex to determine which gender is contributing to postcopulatory selection.

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CHAPTER 2

COMP-1 PROMOTES COMPETITIVE ADVANTAGE OF NEMATODE SPERM

Abstract

Competition among sperm to fertilize oocytes is a ubiquitous feature of sexual reproduction as well as a profoundly important aspect of sexual selection. However, little is known about the cellular mechanisms sperm use to gain competitive advantage or how these mechanisms are regulated genetically. Here, we utilize a forward genetic screen in *C. elegans* to identify a gene, *comp-1*, whose function is specifically required in competitive contexts. We show that *comp-1* functions in sperm to modulate their migration through and localization within the reproductive tract, thereby promoting their access to oocytes. Contrary to previously described models, *comp-1* mutant sperm show no defects in size or velocity, thereby defining a novel pathway for preferential usage. Our results indicate not only that sperm functional traits can influence the outcome of sperm competition, but also that these traits can be modulated in a context-dependent manner depending on the presence of competing sperm.

Introduction

Sexual selection operates at the level of reproductive success to promote traits that improve offspring production (Darwin 1871). It thus influences a wide array of processes that affect not only the likelihood of mating, but also the probability that gametes will interact within a female to form a viable zygote. In many species, a female can mate with multiple males, resulting in competition between male ejaculates, known as sperm competition (Parker 1970). In addition, having multiple mates provides opportunities for a female to influence the outcome, known as cryptic female choice (Eberhard 1996). These postcopulatory forms of sexual selection have driven the diversification of sperm and reproductive tract morphologies as well as the divergence of reproductive proteins, and have likely contributed to reproductive isolation and speciation (Ritchie 2007; Howard et al. 2008; Manier et al. 2013).

Sperm competition is a widespread phenomenon that occurs in species utilizing a wide range of reproductive strategies, and a variety of different patterns of preferential usage, generally referred to as precedence, have been observed (Smith 1984; Birkhead and Møller 1992, 1998). For example, in some species, the first male to mate may show precedence, while in others, the last mate's sperm may win, and the strength of such defensive and offensive abilities varies widely. By their nature, events in the reproductive tract that determine the outcome of competition are difficult to study, so in most cases, the mechanistic basis for a particular precedence pattern is poorly understood. When sperm competition is intense, males often respond by production and transfer of

numerous smaller sperm (Gomendio et al. 1998; Simmons 2001). However, in some cases, sperm may gain an advantage by modulating functional traits, e.g., by increasing migration velocity, promoting retention, or blocking subsequent access to the site of fertilization (Wigby and Chapman 2004; Gomendio and Roldan 2008; Pizzari and Parker 2009).

Due to the difficulty of distinguishing sperm from different ejaculates or of observing sperm directly within the selective environment, indirect assays have often been employed to measure sperm usage. The cell behaviors underlying sperm competition have only been investigated in a few species that are amenable to such analyses, and little is known about the genetic basis for differences in competitive ability among cells. However, *in vivo* imaging studies have recently begun to reveal the cellular mechanisms of sperm behavior in competitive contexts, where multiple males have mated with a female (e.g.,Civetta 1999; Manier et al. 2010; Marie-Orleach et al. 2014). For example, in *Drosophila*, analyses of genetically labeled fluorescent sperm have revealed that stored sperm are highly motile and that modulation of sperm storage, release, and ejection by the female contribute strongly to second-male precedence in that organism (Manier et al. 2010; Lupold et al. 2012). Some genetic loci that affect male reproductive success have recently been identified in *Drosophila* and in mammals (e.g., Fiumera et al. 2005; Sutton et al. 2008; Yeh et al. 2012; Civetta and Finn 2014). Specific seminal fluid components have been shown to play an important role in male competitive advantage by affecting sperm motility and storage, as well as female responses (e.g., Mueller et al.

2008; reviewed in (Avila et al. 2011; Simmons and Fitzpatrick 2012)). However, very few examples are known of genes that function in sperm to control characteristics directly involved in sperm competition. An open question is whether genes exist that specifically regulate competition, without affecting core sperm functions, or whether competitive advantage is always gained by modulating the activity of genes involved in other processes.

The nematode *C. elegans* provides a model system to address the cellular behaviors and molecular pathways that mediate sperm competition. *C. elegans* is a male-hermaphrodite species in which hermaphrodites produce their own self sperm but also can be inseminated by males. In a self-fertilizing context, hermaphrodite self sperm reside in the spermathecae, sperm storage organs where fertilization occurs, and are used with very high efficiency. Typically, more than 99% of sperm go on to fertilize an oocyte (Ward and Carrel 1979). However, if mating occurs, male sperm migrate through the uterus to the spermathecae, where they encounter and must compete with stored self sperm. Importantly, during male-hermaphrodite sperm competition, male sperm are used preferentially (Ward and Carrel 1979; LaMunyon and Ward 1995). Male precedence is very robust, and many crosses result in male sperm exclusively fertilizing oocytes. Simple numerical advantage, seminal fluid factors, and the order of introduction into the reproductive tract have been ruled out as potential causes (Ward and Carrel 1979; LaMunyon and Ward 1994, 1995). Instead, the competitive advantage of *C. elegans* male sperm has been shown to rely on intrinsic differences between male and hermaphrodite sperm cells. While the

form of male and hermaphrodite sperm is the same, male sperm are generally larger than hermaphrodite sperm (LaMunyon and Ward 1999). Consistent with the idea that this is significant, experimental evolution under crossing conditions has been shown to lead to increased size (LaMunyon and Ward 2002). Like those of other nematodes, *C. elegans* sperm move by crawling using a pseudopod, and this motility is required for precedence (Nelson et al. 1982; Singson et al. 1999). Larger sperm crawl faster *in vitro* (LaMunyon and Ward 1998), and male sperm displace self sperm from the walls of the spermathecae (Ward and Carrel 1979). However, male sperm need not fertilize oocytes to outcompete hermaphrodite sperm; mutant males whose sperm are motile, but fertilization-defective, block self progeny production even though their sperm cannot be used (Singson et al. 1999). These data suggest a model for male precedence in which the presence of larger, faster, male sperm leads to the exclusion of self sperm from the fertilization process reviewed in (LaMunyon and Ward 1998; Ellis and Stanfield 2014). Differences in the migration behaviors of male and hermaphrodite sperm could affect the processes of sperm migration towards, retention in, or localization within the spermathecae, where there could be sites especially favorable for sperm-egg interaction (Han et al. 2009). Although many mutants defective for spermatogenesis and/or fertilization have been identified in genetic screens, most mutations affect both male and hermaphrodite sperm equally and none specifically affect male precedence reviewed in (Nishimura and L'Hernault 2010). Thus, the underlying mechanisms, in terms of either cellular behaviors or genetic controls, remain unclear.

Here, we report the use of a genetic screen in *C. elegans* to identify a sperm competition gene. While sperm lacking *comp-1* activity are used efficiently in the absence of competition, *comp-1* sperm are outcompeted by wild type sperm from either hermaphrodites or males, resulting in reduced reproductive success for both *comp-1* mutant males and the hermaphrodites that mate with them. Strikingly, *comp-1* sperm are normal in size. However, they show defects in sperm motility and storage *in vivo*, coupled with context-dependent defects in pseudopodial extension *in vitro*. Thus, *comp-1* regulates sperm-intrinsic functions required to compete both offensively and defensively. These results suggest a model in which *comp-1* functions in sperm to coordinate environmental signals that influence motility related functions required for sperm to compete with one another. Our findings provide key insight into the genetic regulation of sperm competition and suggest that in *C. elegans*, sperm gain advantage by modulating their motility and storage depending on their competitive milieu.

Results

Isolation of a *C. elegans* mutant with defects in male precedence

We took advantage of the male-hermaphrodite reproductive system and robust sperm precedence order of *C. elegans* to perform a forward genetic screen for males with less-competitive sperm. After a wild type male mates with and transfers sperm to a hermaphrodite, his sperm rapidly migrate to the spermathecae and begin fertilizing oocytes, and in ideal conditions, most crosses result in more than 90% cross progeny (Ward and Carrel 1979; LaMunyon and

Ward 1995). However, the underlying mating and sperm transfer behaviors are variable in efficiency, so that in practice, a wide range of cross progeny frequencies are often observed, and some crosses fail altogether (Ward and Carrel 1979 and unpublished observations). Thus, for our screen, we developed a sperm competition assay, using *spe-8; dpy-4* hermaphrodite recipients, that allowed us to exclude crosses for which cross progeny numbers were decreased due to behavioral defects (Figure 2.1A, Materials and Methods). *spe-8* hermaphrodites are self-sterile due to a defect in the ability to activate their self sperm to become motile (L'Hernault et al. 1988). In the absence of mating, they produce no offspring. However, if a male mates with and transfers seminal fluid to a *spe-8* recipient, both the male and self sperm are activated to become motile and fertilization-competent, and since male sperm are superior, they fertilize the vast majority of oocytes (LaMunyon and Ward 1995). The *dpy-4* mutation is recessive and allows discrimination of self progeny from cross progeny on the basis of the Dumpy phenotype. For our assay, we established mating conditions in which most crosses were successful and fewer than five total Dumpy self progeny were produced in the vast majority of cases, providing a readily-scored cutoff for candidate mutants (data not shown).

We performed EMS mutagenesis on a male-producing *him-5* strain (Hodgkin et al. 1979), established lines from individual F2 hermaphrodites, and tested F3 males from each line in the sperm competition assay. We identified one mutant, *me69*, which showed reduced male precedence as compared to the wild type (Figure 2.1B; Stanfield GM, unpublished data). The percentage of cross

progeny that resulted from mating with *me69* mutant males was rarely comparable to that of wild type crosses. However, *me69* mutant hermaphrodites produced a normal number of offspring (Figure 2.1C), setting the *me69* phenotype apart from those of previously identified *spe* mutants, most of which were isolated based on reduction of hermaphrodite fertility but usually affect sperm production in males as well (Nishimura and L'Hernault 2010).

While the use of *spe-8* recipients was critical for our screen, their immotile self spermatids cannot maintain proper positioning within the reproductive tract, resulting in mislocalization and gradual loss of the sperm to the external environment (L'Hernault et al. 1988). To assess precedence of *me69* males in a more natural competitive context, we performed crosses to *dpy-4* hermaphrodites, whose sperm localize appropriately to the spermathecae (data not shown). We placed individual L4 males and *dpy-4* hermaphrodites together for 40 hrs and quantified self and cross progeny generated during this time period. Under these conditions, most matings with wild type control males resulted in at least some cross progeny, and most successful males sired a high fraction of offspring (Figure 2.1D). However, matings with *me69* males resulted in few to no cross progeny during the time frame of this assay. We confirmed that *me69* males were capable of mating and transferring sperm to these hermaphrodites at a high frequency (48-85% of crosses were successful, as compared to 74-100% for wild type), so their poor reproductive success was not simply due to behavioral defects. Rather, *me69* mutant males show postcopulatory defects in sperm usage consistent with a defect in male

precedence.

The *me69* mutation disrupts *comp-1*, a kinase domain gene that functions in sperm

We used meiotic mapping to localize *me69* to a 6.7 Mb interval on chromosome I (Davis et al. 2005; Supplementary Tables 2.1, 2.2). Whole-genome sequencing of the *me69* strain revealed a likely candidate for the causal mutation as a G to A transition in the coding region of *F37E3.3*, an uncharacterized gene that we have renamed *comp-1* for sperm *comp*etition defective (Figure 2.1E). Based on global expression analyses, *comp-1* is expressed in the germ line during time periods that coincide with sperm production: the L4 larval stage in hermaphrodites and in both L4 and adult males (WormBase ; Reinke et al. 2000; Reinke et al. 2004; Ortiz et al. 2014).

The COMP-1 protein contains divergent SH2 and protein kinase-like domains and has been classified within a “unique” subset of *C. elegans* kinases that do not fall clearly within defined families (Manning 2005); it also lacks closely related paralogs within the *C. elegans* genome. It is missing three highly conserved core motifs present in active kinases, including the VAIK motif in the N lobe, the HRD motif in the catalytic loop, and the DFG motif within the activation loop, though it does contain the tripeptide APE motif located within the activation segment (Figure 2.2) (Hanks et al. 1988; Hanks and Hunter 1995; Manning et al. 2002; Nolen et al. 2004; Marchler-Bauer et al. 2011). The absence of these features suggest that the protein is unlikely to have catalytic activity. The *me69*

allele is predicted to result in a glycine to arginine change in a residue that is conserved in all other orthologs identified to date. COMP-1 orthologs are present in other *Caenorhabditis* species as well as in the parasites *H. contortus*, *A. ceylanicum*, and *N. americanus* (WormBase ; Laing et al. 2013; Schwarz et al. 2013; Tang et al. 2014). Although COMP-1 appears to be absent from more distant species (WormBase and unpublished data), it is present in nematodes that utilize male-female as well as male-hermaphrodite reproductive modes.

We obtained a *comp-1* deletion allele, *gk1149*, from the *C. elegans* Deletion Mutant Consortium (Consortium 2012). *gk1149* eliminates a large region of the coding sequence and is likely a null allele. To test if the *me69* and *gk1149* alleles result in a similar male precedence defect, we crossed *gk1149* males to *dpy-4* hermaphrodites and found that *gk1149* mutant males indeed showed a reduction in male precedence as compared to the wild type (Figure 2.1D). Like *me69*, *gk1149* is recessive; crosses with heterozygous *gk1149*/+ males showed a wild type precedence pattern. However, *me69*/*gk1149* heterozygotes had male precedence defects, indicating the two mutations failed to complement one another. To confirm that loss of *comp-1* function is responsible for the male precedence defect, we performed rescue experiments. We generated animals harboring a Mos-mediated single copy insertion (MosSCI) transgene (Frokjaer-Jensen et al. 2008; Frokjaer-Jensen et al. 2012) encompassing a 3.9 kb genomic fragment surrounding *F37E3.3* (Supplementary Tables 2.3, 2.4). This transgene rescued the male precedence defect of both *me69* and *gk1149* males (Figure 2.3), confirming that *comp-1* is the gene affected in these mutants.

To test if *comp-1* function is required in sperm cells, we generated MosSCI transgenes to express it specifically in sperm, using the promoter for the *peel-1* gene (Seidel et al. 2011). We observed full rescue of the male precedence defect in *comp-1(gk1149); Ppeel-1::comp-1* males (Figure 2.1F), indicating that expression of *comp-1* in sperm is indeed sufficient to rescue the male precedence defect. Thus, *comp-1* acts in sperm to promote their preferential usage.

comp-1 activity influences the outcome of male-male sperm competition

Since COMP-1 is highly conserved in both male-hermaphrodite and male-female species (Figure 2.2), we hypothesized that *comp-1* might function in male-male sperm competition. In the standard laboratory strain of *C. elegans* (N2), sequential male matings normally show no precedence pattern, i.e., the first and second males to transfer sperm are equally likely to sire offspring (Ward and Carrel 1979; LaMunyon and Ward 1998). However, sequential matings of males from different wild type strains can show preferential sperm usage patterns (LaMunyon and Ward 1998; Murray et al. 2011), indicating that differences in competitive ability can occur among males in this species. To determine if *comp-1* function influences sperm competition in a male *vs.* male context, we performed sequential matings of wild type and/or *comp-1* males to *fog-2* mutant hermaphrodites, which fail to produce self sperm and are essentially female (Schedl and Kimble 1988). To facilitate assignment of paternity, we used strains

containing a GFP transgene, *mIs11*, for either the first or second sets of crosses, and scored offspring for the presence or absence of fluorescence. In control crosses, in which two wild type males were sequentially mated to hermaphrodites, progeny numbers from the first and second male were variable, but no consistent bias was observed, other than a weak trend in which non-*mIs11* males seemed to be slightly favored over *mIs11*-containing males (Figure 2.4A, Figure 2.5). Similarly, in sequential matings of two *comp-1* males, no precedence order was observed. However, sequential matings of wild type and *comp-1* males resulted in strong precedence for the wild type sperm, regardless of whether wild type males were the first or second mates. Notably, *comp-1* males showed full fertility in crosses to *fog-2* hermaphrodites, which lack their own sperm (Figure 2.4B). These data indicate that *comp-1* males transfer normal numbers of functional sperm, which can be used efficiently when they do not need to compete. However, when other sperm are present, *comp-1* sperm show poor usage. Furthermore, *comp-1* sperm are noncompetitive in both defensive and offensive contexts, suggesting their usage is unrelated to the order of their introduction into the hermaphrodite reproductive tract. Rather, male sperm lacking *comp-1* function appear to have an intrinsic disadvantage as compared to wild type sperm.

comp-1 male sperm are not used until hermaphrodite self sperm are depleted

To investigate the importance of *comp-1* activity for male reproductive success, we sought to determine the nature of the competitive defect of *comp-1* mutant sperm. In particular, we wished to know if *comp-1* sperm usage was delayed as compared to wild type, or if instead it might be generally reduced. To address this, we assayed the long-term kinetics of usage of *comp-1* male sperm within hermaphrodites. We crossed wild type or *comp-1* males to *dpy-4* hermaphrodites for 16 hrs, transferred the recipients at 12 hrs intervals until they ceased egg laying, and counted the total number of self and cross progeny at each time point. Wild type male sperm usage increased rapidly after mating (Figure 2.6A), consistent with previous evidence that male sperm are used preferentially over hermaphrodite self sperm (Ward 1977; Ward and Carrel 1979; LaMunyon and Ward 1995). However, *comp-1* mutant males sired almost no progeny until late in the hermaphrodite lifespan (Figure 2.6A, 2.6B). Furthermore, while mating with wild type males suppressed usage of self sperm, mating with *comp-1* males had no effect on self-progeny production (Figure 2.6C). Thus, *comp-1* males show severe long-term defects in their ability to produce offspring after mating, and hermaphrodites that mate with *comp-1* males produce a decreased number of total offspring (Figure 2.6D). However, although they are initially unsuccessful in fertilizing eggs, at least some *comp-1* sperm are eventually used, indicating that they can remain in the reproductive tract.

Since hermaphrodites make their entire store of self sperm prior to oocyte

production, they gradually run out of self sperm during adulthood. The onset of *comp-1* sperm usage correlated with the depletion of stored hermaphrodite self sperm, suggesting that although *comp-1* sperm remained present from matings that occurred at an earlier time, they were only used once fewer self sperm were present to compete with (Figure 2.6B, 2.6C; compare time points at 52 and 76 hrs). To test if *comp-1* sperm can be used more rapidly when fewer self sperm are present, we aged hermaphrodites until they had used up part or all of their self sperm reservoir, then crossed them to males and assessed the short-term usage of male sperm. In crosses to 12, 24, and 36 hrs post-L4 recipients, which retain moderate levels of self sperm (see Figure 2.6E; "No. remaining sperm"), the number of offspring sired by *comp-1* males increased proportionally to the age of the hermaphrodite, but success was always reduced as compared to wild type males (Figure 2.6E). However, in crosses performed with 48 hrs post-L4 hermaphrodites, which have nearly run out of self sperm, *comp-1* males produced as many offspring as the wild type. Thus, regardless of the length of time they have been resident in the reproductive tract, *comp-1* sperm are unsuccessful specifically in situations where other sperm are present, but can be used in the absence of competition.

comp-1 is expressed and functions to promote sperm usage in both sexes

Global expression studies suggested that *comp-1* is expressed not only in males, but also in hermaphrodites (Reinke et al. 2000; Reinke et al. 2004). We

sought to determine if COMP-1 is indeed present in hermaphrodite sperm and if it shows any differences in localization as compared to male sperm. We first generated transgenic animals carrying a *Pcomp-1::GFP::H2B* transcriptional reporter, in which GFP localizes to the nuclei of *comp-1*-expressing cells. Expression was visible in developing spermatocytes and spermatids in both males and hermaphrodites, and we observed no obvious differences in abundance between the two sexes (Figure 2.7A-D and data not shown).

To determine the localization of COMP-1, we generated worm strains expressing transgenes that contained the full-length *comp-1* coding region fused to either mCherry or GFP. Worms carrying the GFP fusion showed rescue of the male precedence defect, suggesting that the fluorescent tags did not interfere with protein function or localization (Figure 2.8 and data not shown). The COMP-1 fusion proteins displayed a punctate pattern in the cytoplasm of both developing spermatids and mature sperm, where they were restricted to the cell body region (Figure 2.7E-F and data not shown). These punctae were visible in sperm from both males and hermaphrodites (Figure 2.7 and data not shown). To determine whether the COMP-1 protein was localized to a specific subcellular location, we performed colabeling experiments with the vital dye Mitotracker, a marker of mitochondria, and PEEL-1::GFP, which labels the sperm-specific membranous organelles (MOs) (Chen et al. 2000; Seidel et al. 2011). We also examined the phosphatase GSP-3/4, which is involved in cytoskeletal dynamics and shows polarized localization within the pseudopod (Wu et al. 2011). COMP-1 did not colocalize with any of these markers of sperm structure or with the sperm

nucleus (Figure 2.7E-4P), and its absence from the pseudopod suggests that it is not involved directly with cellular locomotion, at least by modulating cytoskeletal dynamics.

The absence of obvious differences between males and hermaphrodites in the expression and localization of COMP-1 raised the question of a potential role for *comp-1* in hermaphrodite self sperm. We thus assayed precedence of wild type and *comp-1* mutant males in crosses to *comp-1* hermaphrodites. Matings of wild type males to *comp-1* hermaphrodites resulted in even higher levels of cross progeny production than those seen in crosses to wild type hermaphrodites, consistent with *comp-1* hermaphrodite sperm having reduced ability to compete (Figure 2.7Q). Interestingly, when *comp-1* males were mated to *comp-1* hermaphrodites, mutant male sperm usage was indistinguishable from that of wild type, suggesting that the male precedence order is regained when *comp-1* sperm compete against each other. These results indicate that *comp-1* functions to promote sperm usage not only in males, but also in hermaphrodites. In addition, factors other than *comp-1* must influence the outcome of competition, as a strong precedence effect can be observed in the absence of its activity in both competing populations of sperm.

comp-1 function is not required for sperm development

One potential explanation for the male precedence defect in *comp-1* mutants was that sperm do not undergo proper spermatogenesis or spermiogenesis necessary to mature into functional sperm. Loss of function of

spe or *fer* genes required for spermatogenesis generally leads to hermaphrodite self sterility and male infertility, and reduction of gene function can result in partial fertility (Kadandale and Singson 2004; Nishimura and L'Hernault 2010). We thus examined available markers of sperm morphology to determine if *comp-1* sperm harbor any general defects. Males and hermaphrodites both produce immotile, spherical spermatids that must be activated to become mature, pseudopod bearing sperm competent for motility and fertility (Wolf et al. 1978). *comp-1* mutant spermatids and sperm appear grossly normal by light microscopy (Figure 2.9A,C; Figure 2.9E,G and data not shown). In addition, several markers of sperm structures localized appropriately in the *comp-1* mutant. As in wild type sperm, mitochondria and membranous organelles were restricted to cell bodies (Figure 2.9A-H) and the GSP-3/4 phosphatase was polarized within pseudopodia (Figure 2.9I-L). The presence of properly polarized sperm structures in mutant sperm indicates that *comp-1* is not required to complete spermatogenesis, nor is it necessary for proper localization of sperm structures in mature sperm cells. These findings are consistent with the absence of fertility or sperm usage defects in *comp-1* animals in the absence of competition.

comp-1 promotes competitive ability independently of cell size

In *C. elegans*, a key factor in conferring male precedence is thought to be the differential size of male and hermaphrodite sperm cells. Male sperm are generally larger than hermaphrodite sperm, correlating with faster crawling speeds *in vitro*, and growth under conditions with a high risk of sperm

competition has been shown to result in increased sperm size (LaMunyon and Ward 1998, 2002). Thus, we investigated the possibility that the precedence defects of *comp-1* males might be due to a reduction in the size of mutant sperm. To assay cell size, we measured spermatids, which are spherical, by obtaining a cross-sectional area through the center of each cell (LaMunyon and Ward 1998). *comp-1* mutant spermatids were variable in size, but the average and distribution of their sizes were indistinguishable from those of wild type spermatids (Figure 2.9M). Therefore, we conclude that loss of *comp-1* does not reduce competitive ability by affecting cell size. Furthermore, *C. elegans* sperm can achieve precedence by a size-independent mechanism.

comp-1 is required for efficient migration to and localization within the spermathecae

In *C. elegans*, sperm are stored and fertilization occurs within the spermathecae (Ward and Carrel 1979). Transferred male sperm must migrate through the uterus and into the spermathecae to be eligible to fertilize oocytes, and male sperm have been observed to displace hermaphrodite sperm from the walls of these structures (Ward and Carrel 1979). We thus examined the ability of *comp-1* mutant sperm to migrate toward and access the spermathecae. We crossed unlabeled hermaphrodites to males either labeled with Mitotracker dye (Kubagawa et al. 2006; Stanfield and Villeneuve 2006) or expressing a sperm *H2B::GFP* reporter, and then examined male sperm positioning at different time points after transfer to the hermaphrodite reproductive tract. Similar to previously

reported analyses of sperm migration (Kubagawa et al. 2006), we divided each proximal gonad arm into four regions: zone 1, near the sperm entry point at the vulva; zone 2, within the uterus; zone 3, the region near the spermatheca; and the spermatheca itself (Figure 2.10A).

By 1-1.5 hrs after transfer, a majority of wild type male sperm had migrated to zone 3 and the spermatheca (Figure 2.10B). Some crosses with *comp-1* males also showed this pattern. However, in many cases, a large percentage of *comp-1* sperm remained in zone 1 and/or zone 2, and accumulation in zone 3 and the spermatheca was reduced (Figure 2.10C). Importantly, wild type and *comp-1* sperm were present in similar, high numbers (an average of 222.4±78.6 for wild type, n=13; an average of 191.1±63.3 for *comp-1*, n=14), and there was no obvious correlation between the number of sperm transferred and their migration efficiency (data not shown). Sperm-specific expression of *comp-1* rescued the migration defect, confirming that the altered migration was due to loss of *comp-1* (Figure 2.11). By 12 hrs after transfer, both wild type and mutant sperm were rarely found in zones 1 and 2, instead localizing to zone 3 and/or the spermatheca (Figure 2.10D; see below). Thus, mutant male sperm show a delay in reaching the spermathecal region. However, their ability to accumulate near the spermathecae at later time points indicates that they are competent to respond to directional cues.

In addition to this delay in migration, we observed a significant decrease in residency of *comp-1* sperm within the spermathecae. At 12 hrs after transfer, when wild type sperm consistently occupied the spermathecae, very few *comp-1*

sperm localized there, even though they were present in zone 3 (Figure 2.10D). Interestingly, by 24 hrs post-mating, mutant male sperm numbers increased within the spermathecae and there was little, if any, difference between wild type and *comp-1* sperm positions (Figure 2.10E). This later time point corresponded to 48 hrs post-L4 adult hermaphrodites, in which self sperm numbers are largely depleted and *comp-1* male sperm start to show increased usage (Figure 2.6B, 2.6E). Taken together, these results suggest that mutant male sperm are not used because they are present at lower numbers in the spermathecae during periods when these structures are occupied with large numbers of self sperm. Since fertilization can occur only within these structures, this defect is likely the primary reason for the reduction in the competitive ability of *comp-1* sperm.

Since *comp-1* functions in both male and hermaphrodite sperm, we also analyzed self sperm in *comp-1* hermaphrodites to assess whether localization defects might still be present in a noncompetitive context. We quantified the position of sperm in different zones in DAPI-stained 24 hrs adult hermaphrodites. In wild type hermaphrodites, most of the sperm resided in zone 3, tightly concentrated just outside of the spermathecae (Figure 2.10F and data not shown); a smaller number was present within the spermathecae. In *comp-1* hermaphrodites, while the majority of sperm were localized within zone 3, fewer sperm resided within the spermathecae as compared to wild type. In addition, some *comp-1* self sperm were mislocalized to zone 2 and occasionally zone 1. Thus, *comp-1* hermaphrodite sperm have minor defects in localization and spermathecal residency that are similar to those of *comp-1* male sperm.

However, these defects do not result in reduced fertility.

comp-1 sperm have context-dependent defects in pseudopodial extension

To probe the cellular basis for the localization defects of *comp-1* sperm, we analyzed their motility using established *in vivo* and *in vitro* assays (Geldziler et al. 2011). Measured immediately after transfer, the migration velocities of *comp-1* sperm within the hermaphrodite uterus were indistinguishable from those of wild type (Figure 2.12A). Furthermore, migrating *comp-1* sperm showed highly directional movement through the uterus towards the spermathecae (Figure 2.12A) and a low reversal frequency, consistent with guided migration (among cells analyzed for motility, only 3/28 wild type cells and 1/25 *comp-1* cells showed one or more reversals during the assay period). The ability of *comp-1* sperm to migrate rapidly *in vivo* suggests that basal motility is not affected in the mutant. However, the difference between wild type and *comp-1* mutant sperm migration patterns could be due to aspects of other migratory behaviors, such as the amount of time individual sperm spend actively migrating through the reproductive tract.

To further analyze the motility of *comp-1* mutant sperm, we dissected spermatids, treated them with the known *in vitro* activators TEA (triethanolamine, a weak base) or Pronase (a protease mixture) (Ward et al. 1983; Shakes and Ward 1989), and sought to measure the velocities of cells crawling on glass slides (Nelson et al. 1982). *comp-1* sperm activated in TEA had extended

pseudopods and were capable of crawling at speeds similar to those of wild type cells (Figure 2.12B-D, Figure 2.13A). *comp-1* sperm treated with Pronase activated at rates similar to the wild type (Figure 2.13B), based on the presence of a pseudopod in the majority of cells. However, the shapes of *comp-1* cells were markedly different from wild type (Figure 2.12E-F and data not shown). Quantification of pseudopod length, using an aspect ratio measurement to normalize for variation in cell size (Batchelder et al. 2011), confirmed that Pronase-treated *comp-1* cells were significantly shorter than either wild type or TEA-treated *comp-1* cells (Figure 2.12B). Since Pronase-treated *comp-1* cells contained distinct cell body and pseudopod regions, with normal localization of organelles (Figure 2.9, Figure 2.12, and data not shown), it is likely that these cells were polarized but failed to extend their pseudopods appropriately. Similar to other amoeboid cells, locomotion of nematode sperm depends on protrusion of the lamellipodium-like pseudopod, adhesion to substrate, and retraction of the cell body (Roberts and Stewart 2000; Bottino et al. 2002). Pseudopod extension defects would be expected to result in altered locomotion and/or interactions with the hermaphrodite reproductive tract, which in turn should affect migration to and occupation of the spermathecae.

Discussion

Taking advantage of the male-hermaphrodite reproductive system of *C. elegans* and its robust natural male precedence order, we have used a genetic screen to identify a sperm competition mutant, *comp-1*. While mutant sperm are

used at normal levels in noncompetitive contexts, they display severe usage defects in all competitive contexts. When wild type sperm are present, *comp-1* sperm are largely absent from the spermathecae and thus are virtually excluded from opportunities to fertilize oocytes. This usage pattern leads to severe defects in male reproductive success for males as well as failure to benefit from outcrossing for hermaphrodites. Consistent with their localization defects *in vivo*, *comp-1* sperm have *in vitro* defects in pseudopodial extension that, like their usage defects, are dependent on context. Together, these phenotypes suggest a cellular role for *comp-1* in modulating the response of sperm to their environment. To our knowledge, *comp-1* is the first gene identified in *C. elegans* to specifically regulate sperm competition and one of few implicated in this process in any organism. Its pattern of conservation in related species suggests a role in male-male sperm competition outside the male-hermaphrodite mode of reproduction used by *C. elegans*. Our findings demonstrate that functional traits can influence the outcome of sperm competition in *C. elegans* in a manner independent of sperm size.

comp-1 and sperm success

For males, reproductive success depends on several functional behaviors of sperm. To fertilize oocytes, sperm must be transferred, become motile, and migrate to the site of fertilization in response to guidance signals (Ward and Carrel 1979; Kubagawa et al. 2006). Overall fecundity depends on the number of sperm that accomplish these behaviors as well as their ability to be stored so as

to ensure long-term usage (Murray and Cutter 2011). We have found that *comp-1* sperm are transferred at rates comparable to the wild type, so they achieve initial entry into the reproductive tract, but they then show varying defects in the ensuing steps (Figure 2.14). Although *comp-1* sperm show delays in migration toward the spermathecae, at least some sperm migrate rapidly and directionally, arguing against a defect in locomotion *per se*. Large numbers eventually accumulate in the spermathecal region, suggesting that they respond to directional cues, but they are generally found outside the spermathecal valve. Once self-sperm stores are depleted, *comp-1* sperm concomitantly gain residency in the spermathecae and begin to fertilize oocytes. Since fertilization occurs only in these structures, it is likely that this localization defect underlies the reduced competitive ability and generally poor reproductive success of *comp-1* males.

Spermathecal occupancy depends on the balance between the rate of entry due to migration and the rate of loss due to displacement by oocytes, which rearrange and even expel a subset of stored sperm as they pass through during ovulation (Ward 1977; Ward and Carrel 1979). Male sperm could thus increase their numbers in the spermathecae either by resisting removal, e.g., by increasing adhesion to the spermathecal walls, and/or by migrating quickly back into the spermathecae, e.g., by increasing their crawling velocity. Defects in pseudopodial extension like those observed *in vitro* for *comp-1* could affect either of these processes, allowing wild type sperm to preferentially associate. Future studies will be necessary to differentiate between the two models, as well as to

characterize the dynamics of sperm behavior in storage.

A unique feature of the *comp-1* phenotype is its dependence on the presence of wild type sperm in recipient hermaphrodites. Do *comp-1* sperm defects occur because wild type sperm behavior is superior, leading to their physical displacement, or does the presence of wild type sperm make *comp-1* sperm inferior, through an indirect mechanism such as signaling? In some organisms, sperm may cooperate by associating with one another to promote fertility or by providing different functions within an ejaculate reviewed in (Higginson and Pitnick 2010), but neither cooperative nor detrimental interactions between sperm have been described for *C. elegans*. Some defects in localization of *comp-1* sperm are observed in the absence of competing cells; a few sperm can be found scattered throughout the uterus, though this mislocalization apparently does not lead to significant reduction in usage or loss from the reproductive tract. These findings are consistent with the migration and localization defects we observed in competitive contexts, and they indicate that *comp-1* defects are not solely induced by the presence of wild type sperm. However, we cannot exclude the possibility that sperm-sperm interactions influence the outcome of competition between *comp-1* and wild type cells.

Several studies have demonstrated a strong association between precedence and cell size in *C. elegans* (LaMunyon and Ward 1998, 1999; Murray et al. 2011). However, loss of *comp-1* has no effect on cell size. Furthermore, *comp-1* activity appears to override the contribution of size, since large *comp-1* male sperm are completely outcompeted by small wild type hermaphrodite

sperm. Interestingly, the normal male precedence order is restored when both male and hermaphrodite sperm lack *comp-1* function, consistent with the idea that the size effect again predominates. Our data thus suggest that multiple activities contribute to precedence and can be independently modulated to affect sperm competitive ability. We note that a mechanism involving altering the activity of COMP-1 is likely to be less costly than production of larger sperm, which is associated with a reduced rate of sperm production (LaMunyon and Ward 1998; Murray et al. 2011).

The cellular role of COMP-1

How does COMP-1 function in sperm to alter motility related behaviors? For crawling cells, locomotion and interaction with substrate are dependent on maintenance of polarity and extension of the lamellipodium, or the pseudopod in the case of nematode sperm reviewed in (Lammermann and Sixt 2009; Reig et al. 2014). *C. elegans* sperm are stably polarized, though the shape and size of their pseudopods is dynamically regulated (Nelson et al. 1982). Markers of the cell body and pseudopod are appropriately localized in *comp-1* sperm, suggesting polarity is not disrupted. However, treatment with Pronase *in vitro*, which is thought to mimic the endogenous male activator (Smith and Stanfield 2011), generates activated cells with severely shortened pseudopods. The sperm cytoskeleton lacks actin and instead consists of Major Sperm Protein (MSP), which generates a network of fibers that drives cell protrusion via its expansion and contraction (Italiano et al. 1999; Roberts and Stewart 2012). In the related

nematode *Ascaris*, MSP filament assembly is mediated by MPOP, a pH-dependent phosphoprotein that is active at the leading edge (LeClaire et al. 2003) and the soluble proteins MFP1 and MFP2 (Buttery et al. 2003). MSP dynamics are also governed in part by the PP1 phosphatase GSP-3/4, which localizes to the proximal pseudopod near the cell body (Wu et al. 2011). Since COMP-1 localizes to the cell body, it seems unlikely to interact directly with the MSP cytoskeleton, but rather might function upstream of locomotion *per se*. COMP-1 contains a protein kinase-like domain, which might suggest a role in signal transduction. Like many other reproductive proteins, it represents a divergent member of its family, and its primary sequence suggests that it is unlikely to be catalytically active. However, in spite of lacking or having reduced enzymatic activity, pseudokinases have been shown to play important roles in cell signaling via interactions with active kinases or their substrates, scaffolding or tethering of signaling complexes, and other mechanisms reviewed in (Reiterer et al. 2014). The punctate localization of COMP-1 within the sperm cell body is intriguing in this context.

Our finding that *comp-1* sperm have reduced pseudopod lengths in an *in vitro* assay fits with their altered patterns of localization *in vivo*. However, measurements of cell velocity indicate that cells lacking *comp-1* are capable of wild type crawling speeds and they eventually accumulate near their appropriate target. Therefore, it is probable that the cellular defects of *comp-1* sperm *in vivo* are less severe than those of Pronase-treated *comp-1* sperm, which have severely shortened pseudopods and should be nearly incapable of movement

(Nelson et al. 1982; LaMunyon and Ward 1999). The *comp-1* phenotype is also distinct from that caused by lack of prostaglandin cues involved in guidance toward oocytes, which leads to a severe reduction in crawling velocity along with loss of directionality (Kubagawa et al. 2006; Edmonds et al. 2010). Thus, *comp-1* sperm are capable of directional migration, though some aspect of sensing or responding to prostaglandins could be impaired. Alternatively, the altered localization of *comp-1* sperm could stem from decreased adhesion to the substrate, leading to a reduced ability to crawl directionally and/or maintain position within the spermathecae. Overall, the context dependence of *comp-1* sperm usage suggests that cellular defects may be limited to a subset of sperm cells or may be manifested only some of the time, for example during interaction with particular substrates within the reproductive tract. Sperm migrate across a variety of tissues including uterine and spermathecal cells and fertilized eggs, each of which could be more or less permissive for migration of *comp-1* mutant cells due to effects on either adhesion or signaling.

comp-1 and reproductive success

The role of *comp-1* in *C. elegans* is evident by the reduction in reproductive success for both sexes in crosses to *comp-1* males. Wild type males who mate successfully can produce hundreds (up to thousands) of offspring (Wegewitz et al. 2008), but *comp-1* males produce very few cross progeny, and these are delayed until other sperm are no longer available. Hermaphrodites mated to wild type males increase their overall progeny

production, but this increase is significantly lower in crosses to *comp-1* males, and few cross progeny are generated. Even in crosses between *comp-1* males and *comp-1* hermaphrodites, where the male precedence order is largely restored, males show reduced success as compared to wild type x wild type matings. Thus, sperm with *comp-1* function should be highly selected for usage when competing with sperm without *comp-1*. In male-female species, we expect that *comp-1* may have a similar function in improving male reproductive success, depending on the rate of polyandry in a given population.

Although self fertilization allows *C. elegans* to propagate without the need to mate and eliminates the cost of producing males, it also leads to reduced genetic variation discussed in (Anderson et al.). The rate of outcrossing in wild populations is estimated to be low, yet males exist, suggesting that some outcrossing may be selected for, or alternatively, that androdioecy has arisen sufficiently recently that the specialized developmental and behavioral characteristics of males have not had time to degrade. Selective pressure has been shown to increase the rate of outcrossing in *C. elegans* in several experimental schemes (Lopes et al. 2008; Morran et al. 2009a; Morran et al. 2009b; Anderson et al. 2010). By promoting the preferential usage of male sperm, COMP-1 should function to increase the genetic diversity of offspring and thus may confer a fitness benefit in situations where adaptation is beneficial (Carvalho et al. 2014).

Sperm competition in *C. elegans*

The outcome of sperm competition depends on the arena in which it occurs, which depends on the specialized reproductive biology and anatomy of the species in question. In particular, differences in the capacity of the sperm storage organ(s), functional characteristics of sperm and seminal fluid, and the degree of sperm mixing lead to distinct patterns of sperm usage (Parker and Pizzari 2010). In *C. elegans*, the spermathecae are somewhat limited as storage sites, which likely reduces the incentive for males to produce and transfer vast numbers of sperm. Instead, the arms race between the sexes leads to males producing sperm that are functionally superior. Once male sperm reach the spermathecae, they are immediately used even though they lack numerical superiority (G.M.S., unpublished data). Interactions between competing ejaculates can be divided into offense, the ability to displace previous sperm, and defense, the ability to block subsequent sperm. Observations of the processes of ovulation, sperm migration, and fertilization in wild type *C. elegans*, as well as the ability of fertilization incompetent sperm to sterilize hermaphrodites, suggest that wild type male sperm most likely block the access of self sperm to the site of fertilization (Ward and Carrel 1979; Singson et al. 1999). However, they fail to block the sperm of another male, as no precedence order is observed in sequential wild type matings (Ward and Carrel 1979; LaMunyon and Ward 1998). *comp-1* male sperm lack the ability to suppress self progeny production, and they also show severe defects in male-male competition whether they are the first or a subsequent mate. Thus, they appear to totally lack the offensive capabilities of

normal sperm and also show defects in defense against new rivals.

COMP-1 is present in both male-hermaphrodite and male-female species of nematodes. Since the male-female reproductive mode is ancestral (Kiontke et al. 2004; Cutter et al. 2008), the function of COMP-1 in sperm competition most likely originated in male-male competition and has been retained in androdioecious species, such as *C. elegans*, where it remains necessary for both male-male and male-hermaphrodite sperm competition. Our results thus establish that *C. elegans* provides a general model to study the molecular mechanisms that underlie sperm competition as well as the interplay between the cell biology of sperm and the forces of sexual selection.

Materials and Methods

C. elegans culture and strains

C. elegans strains were grown at 20°C, except where noted, and fed with OP50 *E. coli* bacteria as previously described (Brenner 1974). All strains were derived from the N2 Bristol wild type strain, with the exception of the CB4856 Hawaiian strain used for mapping. For experiments involving males, *him-5* strains were used as our wild type: *him-5(e1490)* was used for the genetic screen and *him-5(ok1896)* was present in all other strains from which males were obtained (Hodgkin et al. 1979), unless explicitly noted. *comp-1(me69)* was identified in this study and the *comp-1*(*gk1149*) allele was generated by the *C. elegans* Deletion Mutant Consortium (Consortium 2012). Other alleles used for experiments were *spe-8(hc40,hc53)* I, *mIs11[myo-2::GFP, pes-10::GFP and*

gut::GFP], ttTi5605 II, *oxSi221[Peft-3::GFP] II*, *unc-119(ed3) III*, *fem-3(q20*gf*) IV*, *dpy-4(e1166) IV*, *cxTi10816 IV*, *fog-2(q71) V*, and *him-5(e1490, ok1896) V* (Wood and the Community of *C. elegans* Researchers 1988; Maduro and Pilgrim 1995; Frokjaer-Jensen et al. 2008; Frokjaer-Jensen et al. 2012; Meneely et al. 2012).

To generate transgenic strains, Mos-mediated Single Copy Insertion (MosSCI) was used to integrate transgenes at the *ttTi5605* II and *cxTi10816* IV loci (Frokjaer-Jensen et al. 2008; Frokjaer-Jensen et al. 2012).

Genetic screen and identification of *comp-1*

The *me69* mutant was isolated in a screen for males with reduced sperm precedence or fertility. *him-5(e1490)* hermaphrodites were mutagenized using ethyl methanesulfonate (EMS) mutagenesis as described in Wood (1988). Groups of 7-8 P0 hermaphrodites were allowed to self-fertilize; L4 F1 hermaphrodites were picked (25 per plate); and individual L4 F2s were used to establish lines potentially homozygous for newly induced mutations. To assay male precedence, from each viable line, 4-5 L4 males were mated to one *spe-8(hc40); dpy-4* hermaphrodite for approximately 48 hrs, at which time the cross was terminated by removing the hermaphrodite. When all progeny reached at least the L4 stage, mating plates were examined. If at least 5 Dumpy (self) progeny were present, the number of Dumpy (self) and non-Dumpy (cross) progeny were counted. Such lines were retested using the same precedence assay as before. Approximately 3400 mutagenized lines were tested and 16 lines

were recovered as homozygous mutants. Of the 16 lines, six lines had normal gonadal and sperm morphology, consistent with a precedence-specific defect. The *me69* mutant was among those 6 lines.

To map *me69*, CB4856 (Hawaiian) males were crossed to *me69; him-5* hermaphrodites, F1 males were crossed back to *me69; him-5* hermaphrodites, and individual F2 males were tested for the male precedence defect. Each male was recovered into lysis buffer and males scoring as mutant were assayed for a centrally-located SNP on each chromosome (Wicks et al. 2001). Linkage was detected to chromosome I and additional SNPs were scored in individual males to narrow *me69* to a 6.7 Mb region between WBVar00240399 and WBVar00240414 (Tables 2.1, 2.2) (WormBase; Jakubowski and Kornfeld 1999). To identify the gene affected in *me69*, whole genome sequence was obtained from the strain isolated in our genetic screen. Of 45 variations in the *me69* region, 24 were consistent with EMS, seven affected coding regions, and only one affected a gene (*F37E3.3*) showing sperm-enriched gene expression.

Molecular biology

Molecular biology was performed according to standard protocols. The Multisite Gateway Three-Fragment Vector Construction Kit (Life Technologies) was used to construct donor plasmids. Fragments were then recombined into the MosSCI destination vectors pCFJ150 or pCFJ212 (Frokjaer-Jensen et al. 2008; Frokjaer-Jensen et al. 2012). For constructs in which two fragments were ligated by PCR, fusion PCR was performed as in Hobert (2002). Primers used for

generating constructs are listed in Table 2.3 and plasmid construction strategies are summarized in Table 2.4.

Fertility and sperm competition assays

To measure hermaphrodite fertility, L4 hermaphrodites were individually placed on a freshly seeded lawn and moved to a new plate every 24 hrs until eggs were no longer laid. To measure male fertility, L4 males were crossed in a 1:1 ratio to L4 *fog-2* females for 24 hrs. The males were then removed and the females were transferred every 24 hrs until egg laying ceased. Progeny were counted after reaching at least the L4 stage. The variability in cross progeny number observed in these experiments is typical of this assay and is generally attributed to variation in mating, sperm transfer, and/or sperm loss (Murray et al. 2011).

To test short-term male precedence, L4 males and *spe-8(hc53); dpy-4* or *dpy-4* L4 hermaphrodites were placed together in a 1:1 ratio onto plates with freshly seeded lawns. After 40 hrs, both parents were removed. Upon reaching adulthood, offspring were scored as either Dumpy (self) or non-Dumpy (cross) progeny and counted. To test long-term male precedence, animals were allowed to mate for 16 hrs, hermaphrodites were transferred to fresh plates every 12 hrs, and self and cross progeny were scored as described above. To test the effect of hermaphrodite age on male precedence, 12, 24, 36, or 48 hrs post-L4 hermaphrodites were crossed to 24 hrs post-L4 males for 24 hrs, both parents were removed, and the number of self and cross progeny were scored as

described above. To estimate the number of self sperm remaining in the hermaphrodite reproductive tract at each time point, the number of progeny from unmated hermaphrodites picked in parallel was counted.

Male-male competition assays were performed by placing 24 hrs post-L4 adult males ("first" males) with 24 hrs post-L4 adult *fog-2(q71)* hermaphrodites for 3 hrs in an 8:6 ratio of males to hermaphrodites. The hermaphrodites were allowed to recover for 1 hr, and those lacking visible embryos in their uteri were removed from the plate. The "second" males, 28 hrs post-L4, were then placed with the hermaphrodites and allowed to mate for 3 hrs. Individual hermaphrodites were then moved to fresh plates, allowed to lay eggs for 16 hrs, then transferred. To distinguish progeny of first and second mates from one another, second-male strains harbored an integrated GFP transgene, *mIs11*; to control for possible marker-specific effects, experiments were repeated with the *mIs11* strains as first males. Progeny generated 0-16 hrs after mating were quantified. Subsequent progeny were scored for GFP, and only plates that contained both GFP-positive and GFP-negative offspring were included in analyses.

For all experiments involving measurements of progeny numbers, wild type and mutant animals were tested in parallel to control for variations in temperature and/or media quality that can affect mating and fertility. Each experiment was repeated 2-4 times, and figures show representative results.

Microscopy and immunohistochemistry

To release spermatids, adults were dissected in a drop of sperm medium (SM; 50mM HEPES pH7.8, 50mM NaCl, 25mM KCl, 1mM $MgSO_4$, 5mM $CaCl_2$, and 10mM dextrose). Virgin 48 hrs post-L4 males grown at 20°C were used. Where necessary, spermatids were incubated in SM containing 60mM TEA or 200µg/ml Pronase to induce activation into motile sperm (Shakes and Ward 1989). Antibody staining followed a protocol similar to that in Wu (2011). Briefly, an equal volume of 4% paraformaldehyde in SM was added to the dissected cells. The slides were then incubated in a humid chamber for 5 mins, freeze-cracked on a metal block placed in liquid nitrogen, incubated in 95% ethanol for 1 min, and washed with PBST (phosphate-buffered saline pH 7.2, 0.5% Triton X-100, 1mM EDTA). Antibody incubations were performed for 16 hrs at 4°C with rabbit anti-GSP-3/4 (rb1496, 1:500) (Wu et al. 2011) and 1 µg/mL DAPI (4',6-diamidino-2-phenylindole) and for 2 hrs at 4°C in goat anti-rabbit AlexaFluor 488- or AlexaFluor 568-labeled IgG (Life Technologies) at 1:1000; antibodies were diluted and washes were performed in PBST with 1% BSA (bovine serum albumin). Slides were mounted with VectaShield (Vector Laboratories). Confocal images were acquired using an Olympus FV1000 confocal microscope.

in vivo sperm migration and localization assays

To analyze localization of male sperm up to 2.5 hrs after transfer, Mitotracker Red CMXRos (Life Technologies) was used to label male sperm as in Stanfield (2006). To analyze sperm localization more than 2.5 hrs after

transfer, virgin 24 hrs post-L4 males carrying the *Pcomp-1::GFP::H2B* transcriptional reporter were mated for 45 mins to 24 hrs post-L4 N2 hermaphrodites anesthetized in 0.1% tricaine and 0.01% tetramisole (McCarter et al. 1997), and males were then removed. At 12 hrs or 24 hrs postmating, images of each recipient were captured in multiple focal planes to capture an entire gonad arm. Analysis of sperm position was performed as in Edmonds (2010). Depending on the experiment, either all GFP-positive male sperm in a gonad arm were counted, or those in the focal plane that had the most sperm in the spermatheca were counted. To analyze localization of self sperm, 24 hrs post-L4 hermaphrodites were fixed with Carnoy's fixative (Ellis and Horvitz 1986) and stained with DAPI at 1µg/mL in M9. Image collection and data analysis were performed as for male sperm.

To measure *in vivo* velocity, images of migrating cells were collected as in Kubagawa (2006) using an AxioImager M1 microscope, Axiocam camera, and Axiovision software (Zeiss). Cells within Zone 2 that moved for at least four consecutive frames were analyzed using the plugins Manual Tracking and Chemotaxis and Migration Tool (Ibidi) in ImageJ (Schneider et al. 2012).

in vitro sperm morphology and function assays

Sperm were activated *in vitro* as described previously and DIC (differential interference contrast) images were captured every 60 secs for 30 mins (Shakes and Ward 1989; Fenker et al. 2014). To quantify activation, sperm were scored for the presence of either spikes or a pseudopod at 30 mins after adding

Pronase. Nonactivated sperm from control slides lacking activator were used to measure spermatid size. Aspect ratio was measured by dividing the total length of the pseudopod and cell body by the width of the cell body. The center of the cell body was determined by fitting a circle or ellipse around the cell body and finding the center of that object. The length was then determined by drawing a line from the tip of the pseudopod to an edge of the cell body, with the line dissecting the center of the cell body, and the width of the cell was measured by drawing a line perpendicular to the length and dissecting the center of the cell body. Velocity was measured in TEA-activated sperm that moved for at least three consecutive frames. Measurements were obtained using ImageJ (Schneider et al. 2012).

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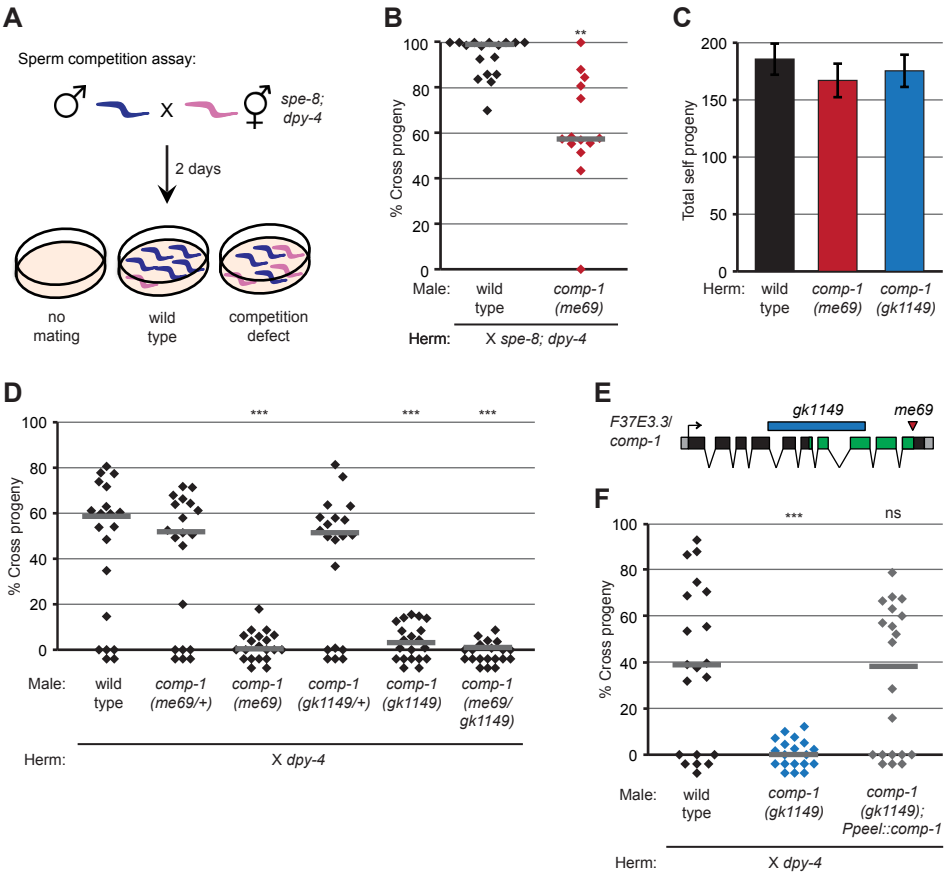
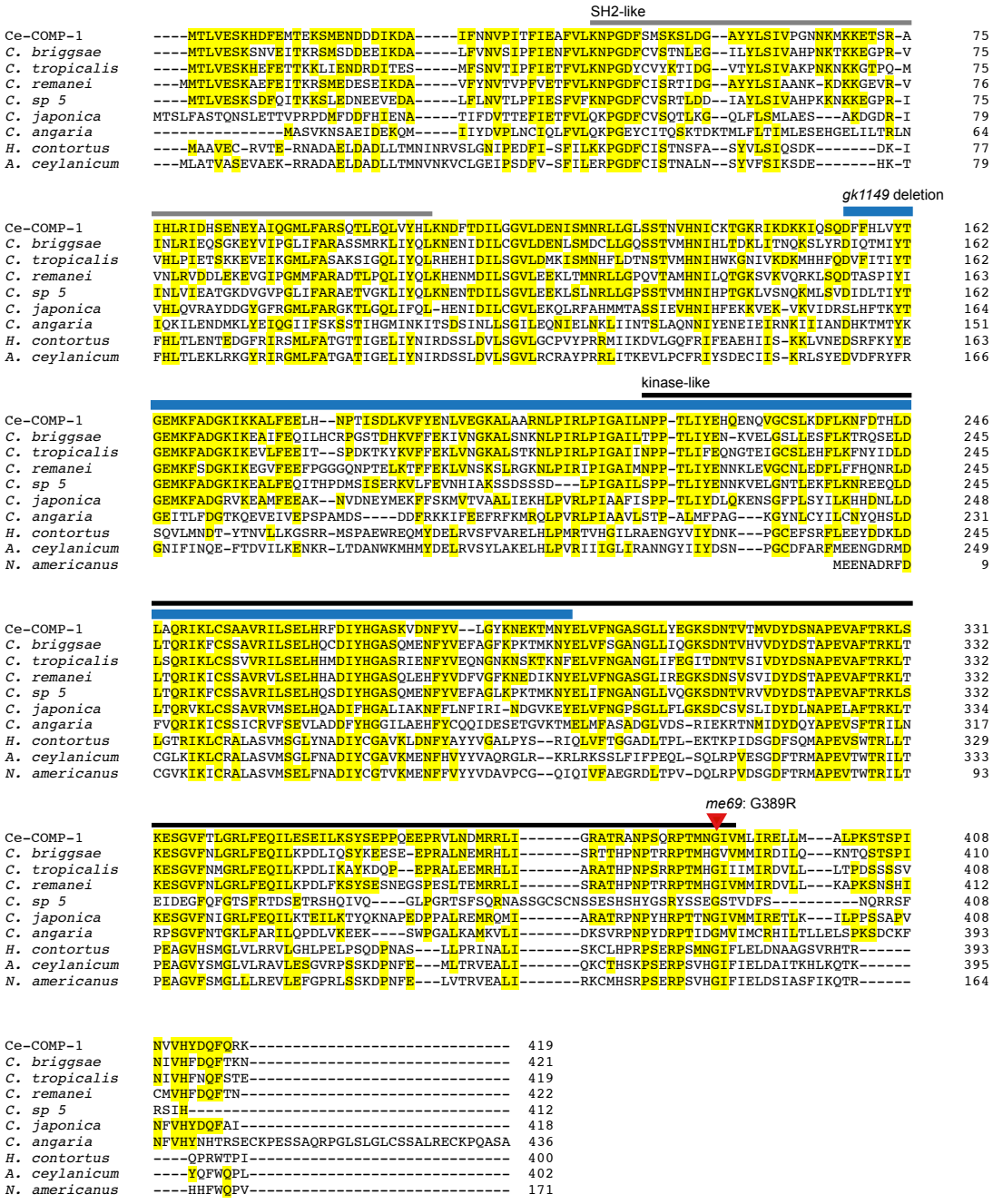


Figure 2.1. Isolation of the male precedence mutant *me69* in a genetic screen. (A) Screening assay for mutants with reduced male precedence, showing outcomes for mating failure, mating by wild type males, and mating by males with less competitive sperm. (B) *me69* males have decreased precedence in the screen assay. Males were mated to *spe-8(hc53); dpy-4* hermaphrodites, and offspring were scored as Dumpy (self) or non-Dumpy (cross) progeny. (C) *me69* and *gk1149* mutant hermaphrodites have normal self fertility. Total self progeny of unmated hermaphrodites were counted. Error bars, 95% confidence intervals; P>0.05 (Student's t test). (D) Mutants for *comp-1* have defects in male precedence. Males were mated to *dpy-4* hermaphrodites, and offspring were scored as Dumpy (self) or non-Dumpy (cross). (E) Schematic of the *F37E3.3* gene showing the kinase-like domain (green) predicted by the Conserved Domains Database (Marchler-Bauer et al., 2011) and the locations of the *me69* and *gk1149* alleles. Boxes, exons; black, coding regions; grey, 5' and 3'UTRs. (F) Expression of the *F37E3.3* gene in sperm rescues the *comp-1* male precedence defect. Male precedence was assayed for *comp-1(gk1149); jnSi168[Ppeel::-comp-1]* and control strains as in Figure 2.1D. (B,D,F) Each point represents the result of an individual cross; lines indicate medians. ***, p<0.001; **, p<0.01 (Kolmogorov-Smirnov test; all comparisons are to wild type). In addition to the genotypes shown, all males were homozygous (B,C,F) or heterozygous (D) for *him-5(ok1896)*, and control strains in (F) harbored *oxSi221*.

Figure 2.2. COMP-1 is highly conserved within the *Caenorhabditis genus* and present in related parasitic species. Alignment of *C. elegans* COMP-1 with its orthologs from other nematode species. Yellow highlighting represents amino acids conserved with COMP-1. Bars above the sequence indicate the positions of the divergent kinase-like (black) and SH2-like (grey) domains, as predicted in the Conserved Domains Database (Marchler-Bauer et al. 2011); an alternate prediction for the kinase-like domain includes amino acids 123-394 (Manning 2005, see www.kinase.com). Positions of the *me69* and *gk1149* alleles are shown in red and blue as in Figure 2.1E. Previous annotation (WormBase) of the *C. japonica comp-1* region predicted two overlapping gene models encoding proteins similar to the N and C terminus of COMP-1; our new predicted Cja-COMP-1 fuses JA64544 and CJA40432 and maximizes similarity with Ce-COMP-1 and the other orthologs. Other accession numbers: Acey_s0303.g1899.t2 (PRJNA231479), Cang_2012_03_13_00293.g8644.t2 (PRJNA51225), CBP27128, RP29840, Csp5.Scaffold_00675.g14294.tt (PRJNA194557), Csp11.Scaffold630.g17815.ti (PRJNA53597), HCOI01934100.t1 (PRJEB506), and NECAME_15795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kinase-like
me69: G389R

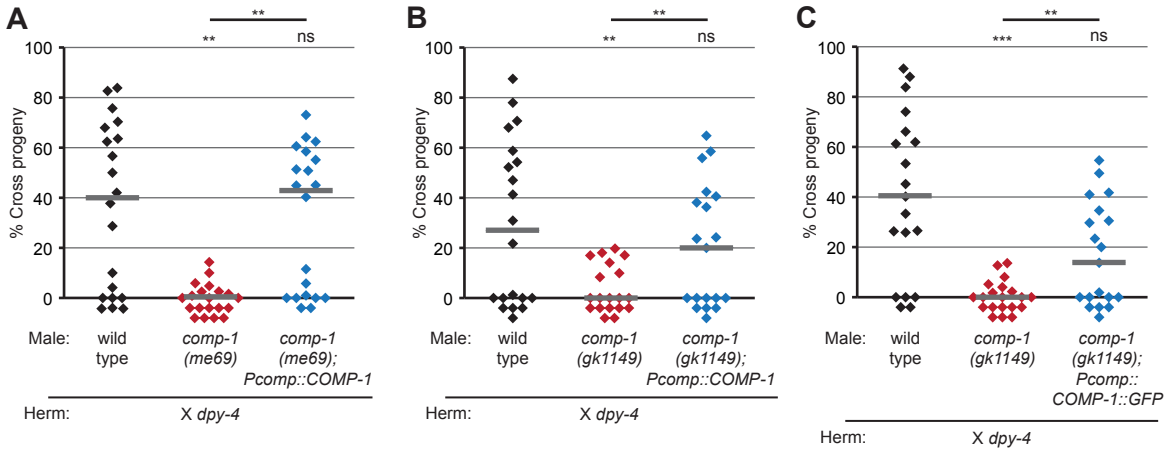

Figure 2.3. COMP-1 transgenes rescue the male precedence defects of *comp-1* mutants. (A,B) The *jnSi109[Pcomp-1::COMP-1]* transgene, which contains a 3.9 kb region surrounding *F37E3.3*, rescues the precedence defect of (A) *comp-1(me69)* and (B) *comp-1(gk1149)* males in crosses to *dpy-4* hermaphrodites. (C) Expression of COMP-1::GFP rescues the precedence defect. *comp-1(gk1149)*; *jnSi171[Pcomp-1::COMP-1::GFP]* males have a wild type precedence pattern in crosses to *dpy-4* hermaphrodites. Precedence assays were performed as in Figure 2.1D. ***, P<0.001; **, P<0.01; ns, not significant

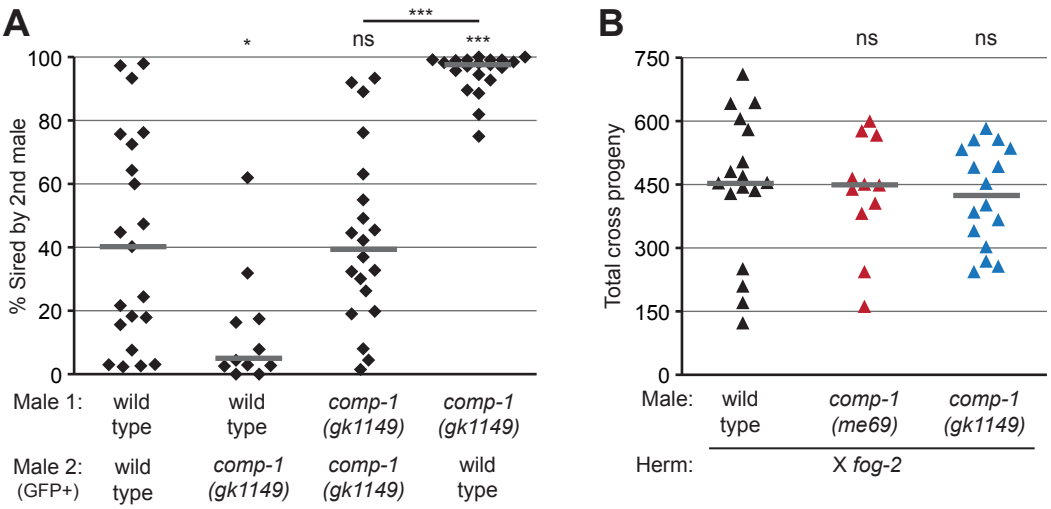

Figure 2.4. The *comp-1* mutant has defects in male-male sperm competition. (A) *comp-1* male sperm are outcompeted by wild type male sperm. Wild type and/or *comp-1(gk1149)* males were mated sequentially to *fog-2* hermaphrodites; second-mated males harbored the transgene mIs11(GFP+). Offspring were scored for GFP, and the percentage of GFP-positive progeny produced 0-16 hrs after second-male mating is shown. (B) *comp-1* mutant males have wild type levels of fertility in the absence of competition. Males were crossed to *fog-2* hermaphrodites and total progeny were counted. (A,B) Individual data points are shown; lines indicate medians. *, P<0.05; ***, P<0.001; ns, not significant (Kolmogorov-Smirnov test; comparisons are to wild type unless indicated by a line linking the two data sets).

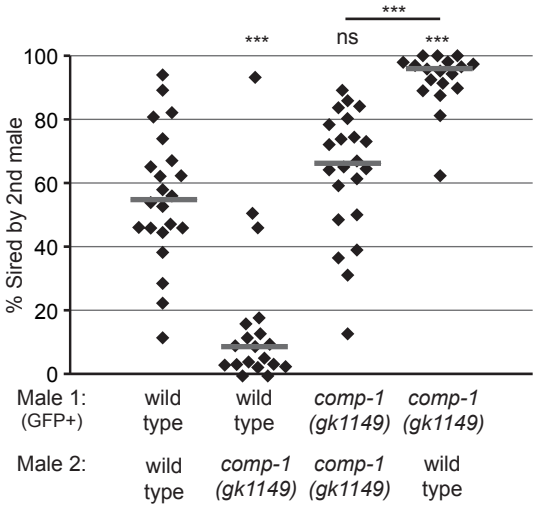

Figure 2.5. The *comp-1* mutant has reduced male precedence. *comp-1* male sperm are outcompeted by wild type male sperm. Wild type and/or *comp-1(gk1149)* males were mated sequentially to *fog-2* hermaphrodites; first-mated males harbored the transgene mIs11(GFP+). Offspring were scored for GFP, and the percentage of GFP-positive progeny produced 0-16 hrs after second male mating is shown. GFP-marked males show an apparent slight disadvantage, which is observed consistently but is not statistically significant. Lines indicate medians. ***, P<0.001; ns, not significant (Kolmogorov-Smirnov test).

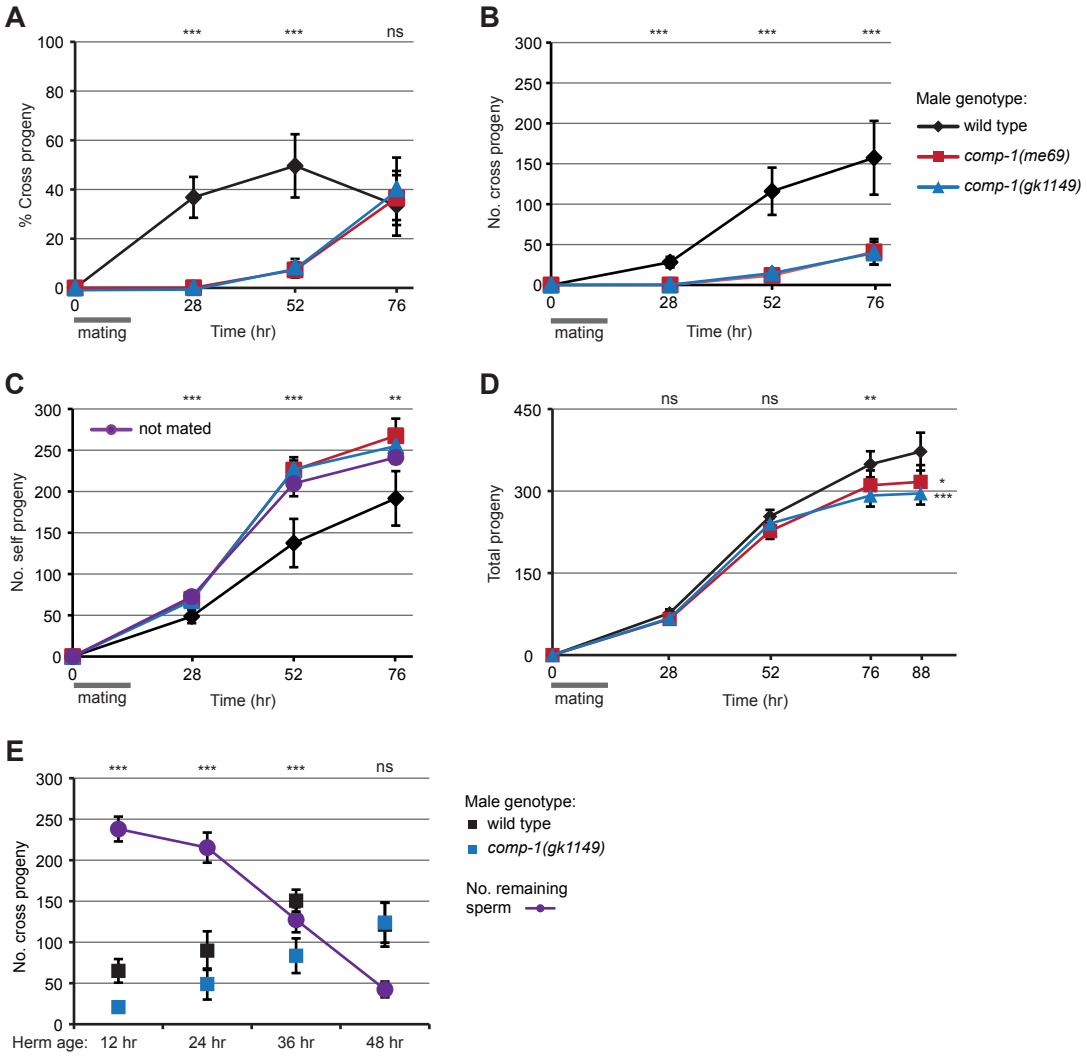

Figure 2.6. *comp-1* male sperm have long-term precedence defects. (A) Crosses with *comp-1* males result in a low percentage of cross progeny. (B) The number of cross progeny sired by comp-1 increases at late time points. (C) Crosses with *comp-1* males do not suppress production of self progeny. Purple line indicates self progeny of unmated hermaphrodites. (D) Crosses with *comp-1* males result in decreased progeny numbers as compared to those with wild type males. (A-D) Males were crossed to *dpy-4* hermaphrodites for 16 hrs (grey line); progeny were collected throughout the recipients' reproductive lifespans and scored as self or cross progeny. All graphs are from a single data set that is representative of three repeats. (E) *comp-1* male sperm are used at wild type levels in crosses to sperm-depleted hermaphrodites. Males were crossed to staged *dpy-4* recipients for 24 hrs and progeny generated during the mating period were scored as self or cross progeny. "No. remaining sperm" indicates the number of self sperm present within recipients at each stage, inferred from brood counts of unmated *dpy-4* hermaphrodites performed in parallel. Data points indicate averages; error bars, 95% confidence intervals. **, P<0.01; ***, P<0.001; ns, not significant (Kolmogorov-Smirnov test, comparing wild type to each *comp-1* mutant).

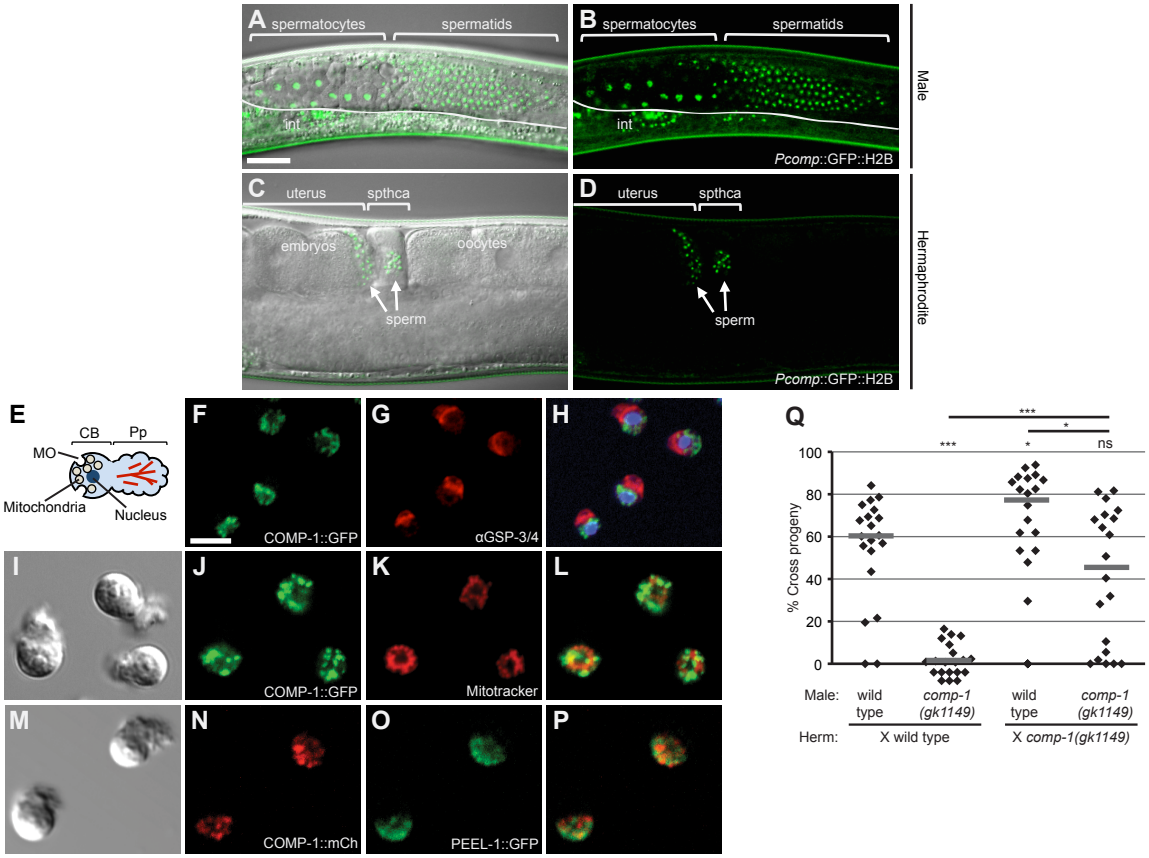

Figure 2.7. COMP-1 is expressed and functions in sperm of both males and hermaphrodites. (A-D) Images of *jnSi118*[Pcomp-1::GFP::H2B] adult males (A,B) and hermaphrodites (C,D), which express the comp-1 reporter in developing sperm. int, intestinal autofluorescence. Scale bar (A-D), 30 µm. (E) Schematic of structural organization of spermatozoa. (F-H) COMP-1 does not colocalize with GSP-3/4, which is in the pseudopod. Images of *jnSi171*[COMP-1::GFP] spermatozoa fixed and stained with α-GSP-3/4 antibody (red) and DAPI (blue). Scale bar (F-P), 5 µm. (I-L) COMP-1 does not colocalize with mitochondria. Images of *jnSi171*[COMP-1::GFP] spermatozoa stained with Mitotracker. (M-P) COMP-1 does not colocalize with PEEL-1::GFP, which is at the membranous organelles. Images of *jnSi143*[COMP-1::mCherry]; *jnSi177*[PEEL-1::GFP] spermatozoa. (Q) *comp-1* functions in both male and hermaphrodite sperm. Wild type and *comp-1(gk1149)* males were tested against wild type and *comp-1(gk1149)* hermaphrodites in the short-term precedence assay. Lines indicate medians. *, P<0.05; ***, P<0.001; ns, not significant, Kolmogorov-Smirnov test.

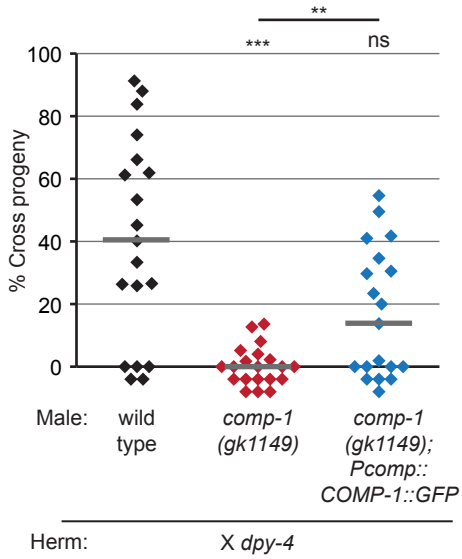

Figure 2.8. A COMP-1::GFP transgene rescues the male precedence defects of *comp-1* mutants. Expression of COMP-1::GFP rescues the precedence defect. *comp-1(gk1149); jnSi171[Pcomp-1::COMP-1::GFP]* males have a wild type precedence pattern in crosses to *dpy-4* hermaphrodites. Precedence assays were performed as in Figure 2.1D. ***, P<0.001; **, P<0.01; ns, not significant (Kolmogorov-Smirnov test). Lines indicate medians. In addition to the indicated genotypes, control strains contained the transgene o*xSi221[Peft-3 ::GFP].*

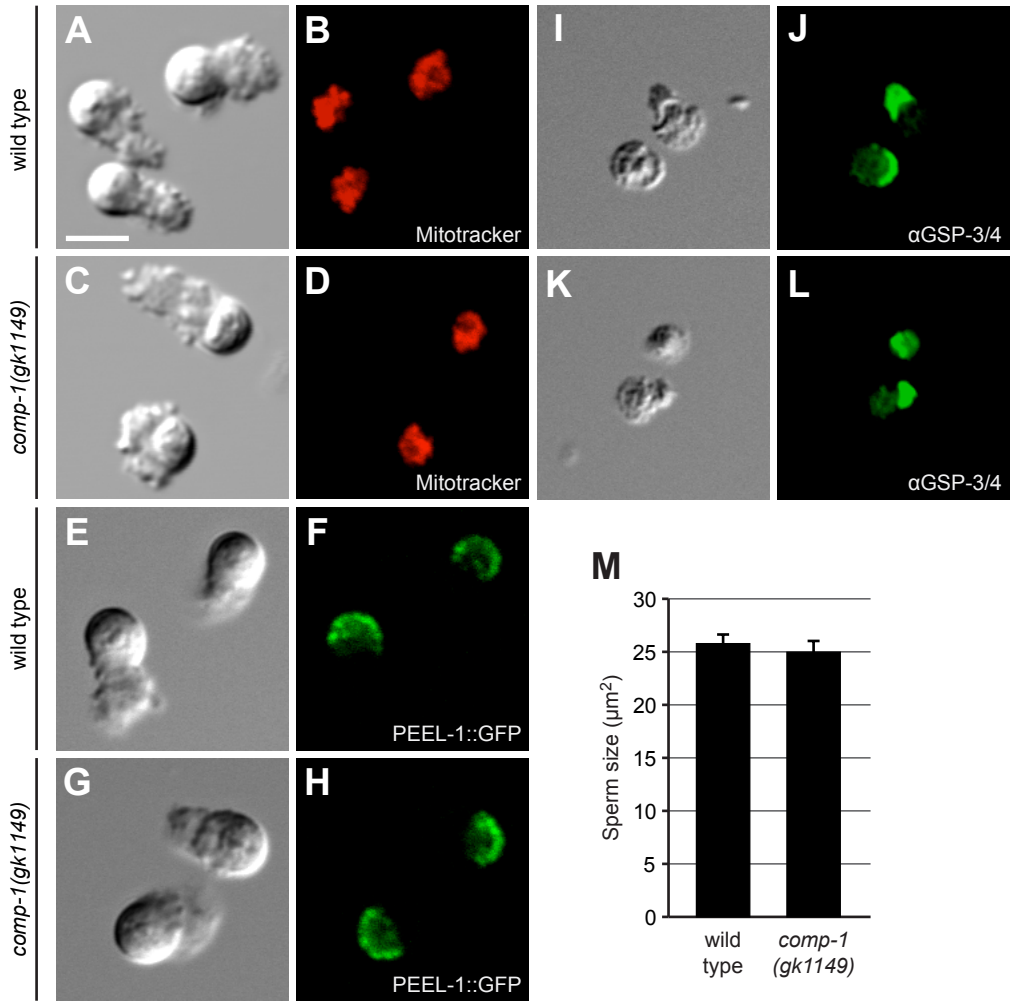

Figure 2.9. *comp-1* sperm have normal organization and size. (A-D) Wild type (A,B) and *comp-1(gk1149)* (C,D) spermatozoa stained for mitochondria using Mitotracker. (E-H) Wild type (E,F) and *comp-1* (G,H) spermatozoa expressing PEEL-1::GFP (membranous organelles). (I-L) Wild type (I,J) and *comp-1(gk1149)* (K,L) spermatozoa fixed and stained with α-GSP-3/4 antibody (green). (A-L) Scale bar, 5 μm. (M) *comp-1* male spermatid size is not significantly different from wild type. Cross-sectional areas through the center of spermatids were measured. Error bars, 95% confidence interval; p=0.41, Student's t test.

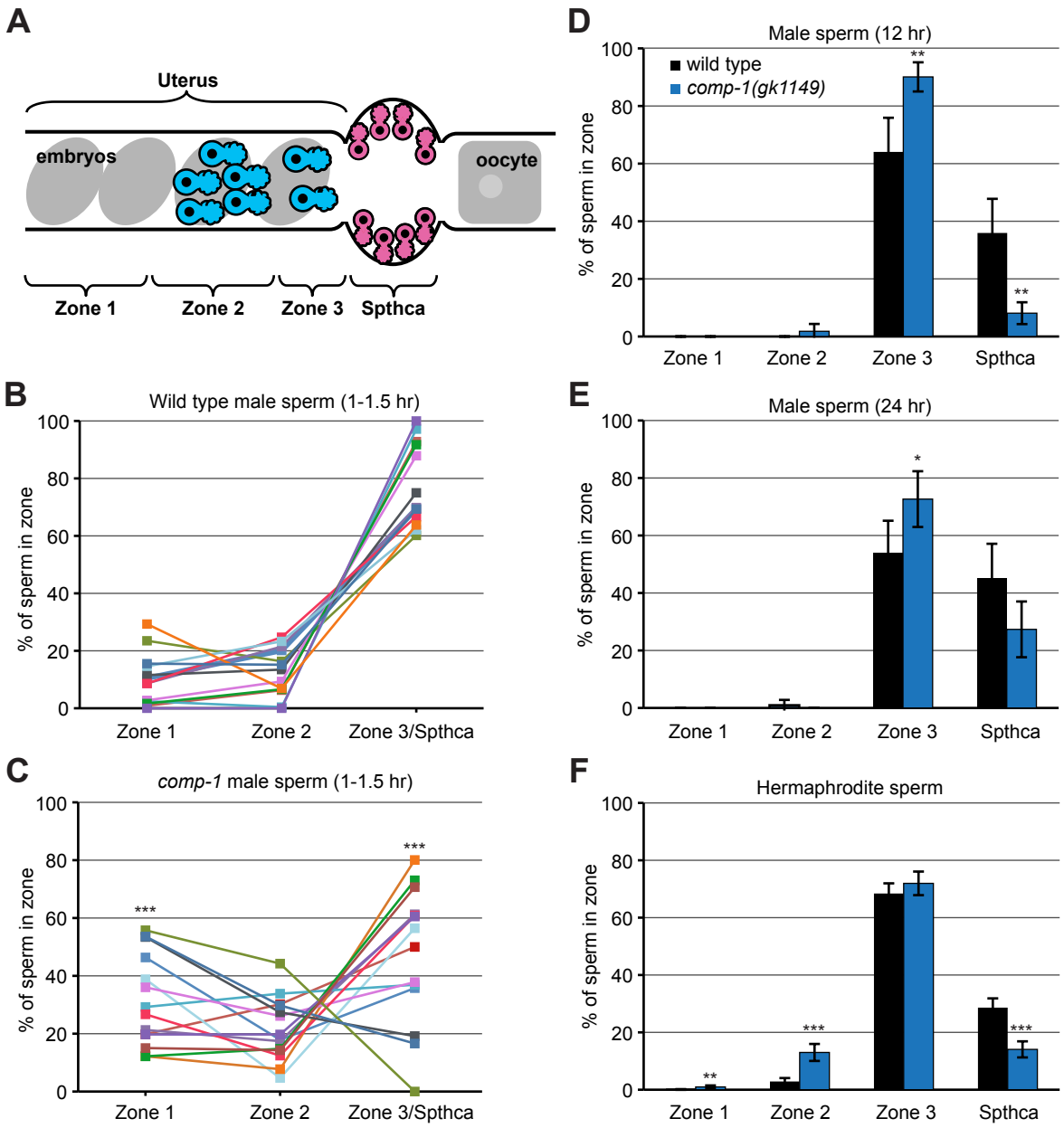

Figure 2.10. *comp-1* male sperm have defects in migration and spermathecal accumulation. (A) Schematic of hermaphrodite gonad arm showing zones used to quantify sperm position. (B,C) Localization of wild type (B) and *comp-1(gk1149)* (C) Mitotracker-labeled male sperm 1-1.5 hrs after transfer to hermaphrodites. Percentage of total male sperm is shown. (D,E) Localization of *jnSi118[GFP::H2B]* male sperm 12 hrs (D) and 24 hrs (E) after transfer to hermaphrodites. Percentage of male sperm in the focal plane with maximum sperm in the spermatheca is shown. (F) Localization of hermaphrodite self sperm in 24 hrs post-L4 unmated hermaphrodites. Animals were stained with DAPI to facilitate counting of sperm cells. Percentage of total hermaphrodite sperm is shown. Error bars, 95% confidence intervals. *, p<0.05; **, p<0.01; ***, p<0.001; Student's t test.

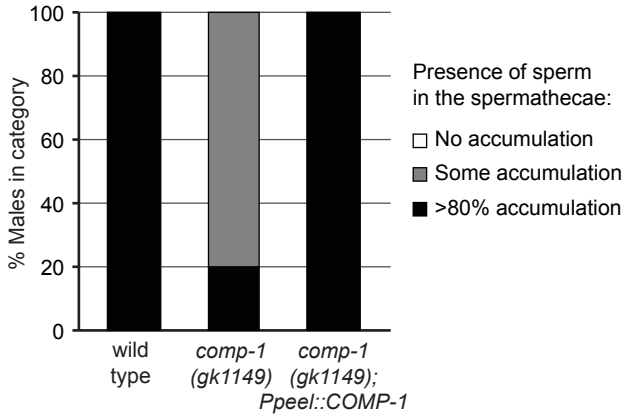

Figure 2.11. Sperm-specific expression of *comp-1* rescues the migration defect. *comp-1(gk1149); jnSi168[Ppeel-1::comp-1]* mutant male sperm accumulate in the spermathecal region at rates similar to wild type. Mitotracker-labeled males were crossed to *dpy-4* hermaphrodites and the position of transferred sperm was assessed 2-2.5 hrs after mating. Sperm localization was scored by eye as more than 80% accumulation, some accumulation, or no accumulation in the zone 3/spermatheca region. n=12-15 for each genotype; data are representative of four repeats.

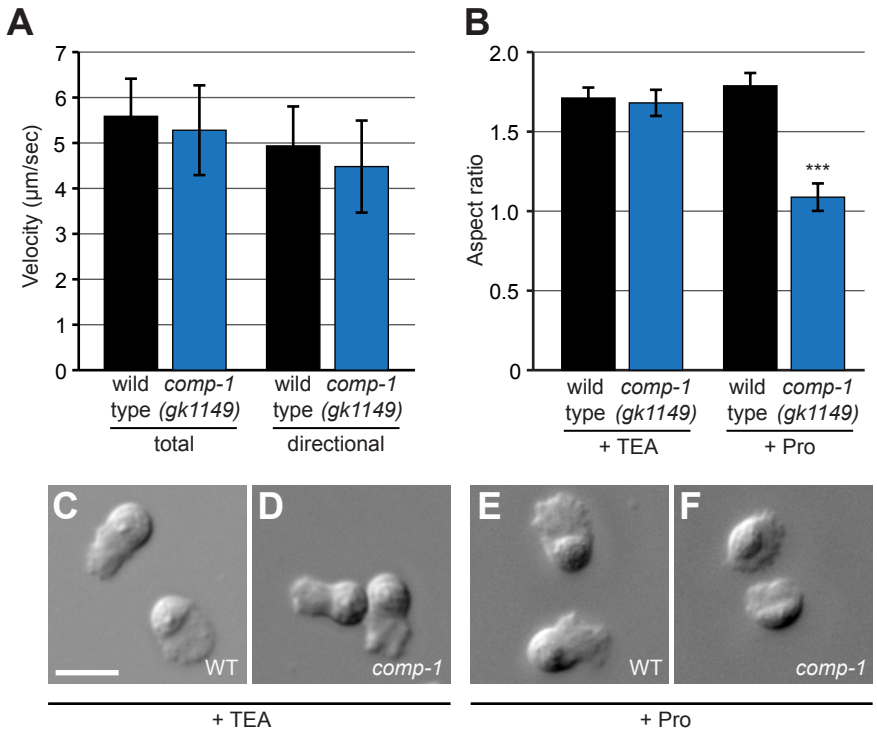

Figure 2.12. *comp-1* male sperm can migrate normally but have context-dependent defects in cell morphology. (A) *comp-1(gk1149)* sperm can migrate *in vivo* at speeds equivalent to wild type sperm. Mitotracker-labeled males were crossed to N2 hermaphrodites and time-lapse images of sperm migrating through zone 2 were collected. Velocity and directional velocity toward the spermatheca were measured using ImageJ. (B-F) *comp-1(gk1149)* spermatids show reduced pseudopodial extension after activation by Pronase. (B) Quantification of aspect ratios of wild type and *comp-1(gk1149)* sperm treated with either TEA or Pronase. (C-F) Representative images of wild type (C,E) and *comp-1(gk1149)* (D,F) sperm treated with TEA (C,D) or Pronase (E,F). Error bars, 95% confidence interval; ***, P<0.001, Kolmogorov-Smirnov test. Scale bar, 5 µm.

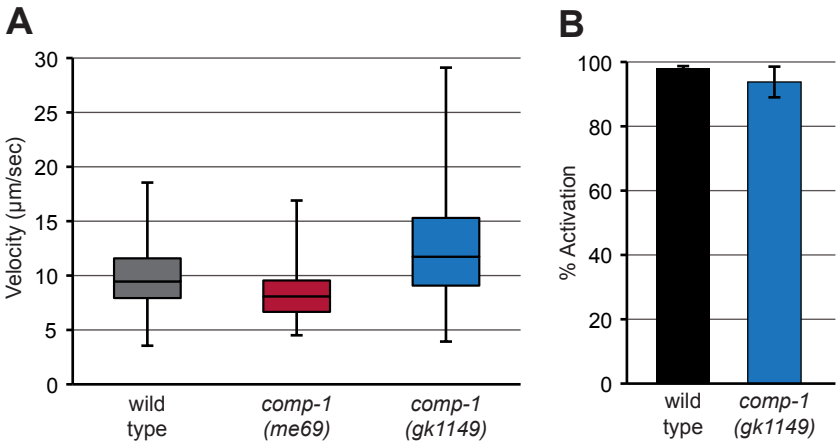

Figure 2.13. *comp-1* sperm can crawl and be activated *in vitro***.** (A) *comp-1* sperm can crawl at wild type velocities *in vitro*. Wild type and *comp-1(gk1149)* spermatids were treated with TEA for 30 mins and velocity was obtained from time-lapse images collected every 30 secs. As we observed a high level of variability among different samples for each genotype, the range of observed values is shown using a box-and-whiskers plot. For each genotype, n=5-6 samples, 65-130 cells. (B) c*omp-1* sperm activate in Pronase. Wild type and c*omp-1(gk1149)* spermatids were treated with Pronase for 30 min and scored for activation based on the presence or absence of a pseudopod. Error bars, 95% confidence intervals.

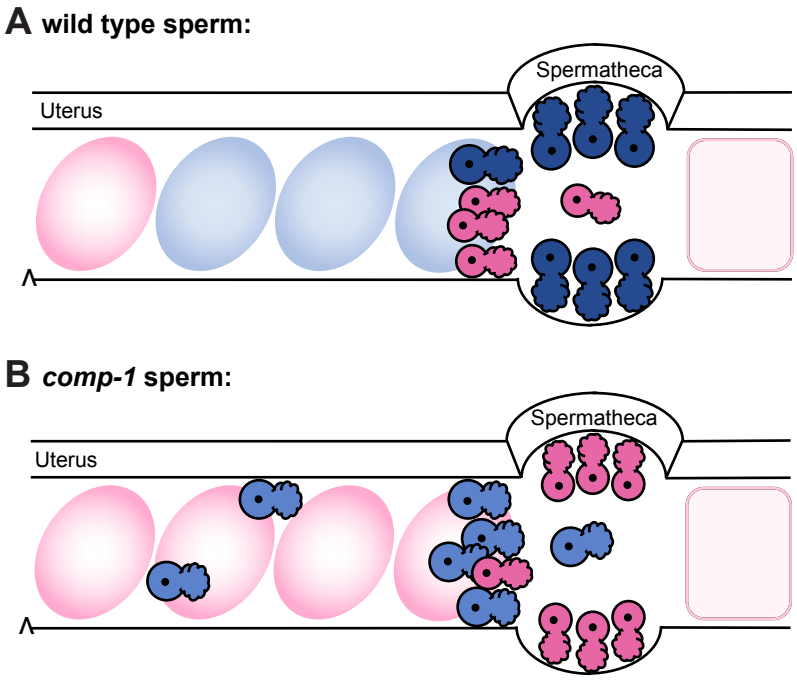

Figure 2.14. Model: *comp-1* sperm have localization defects that result in failure to compete with wild type sperm. (A) Wild type male sperm (blue) migrate to the region of the spermathecae, where they displace hermaphrodite self sperm (pink) and preferentially fertilize oocytes. (B) *comp-1* mutant male sperm (light blue) migrate to the spermathecae but remain outside while wild type sperm (pink) are present, and are thus excluded from opportunities to fertilize oocytes. They also show delayed migration to the spermathecal region and increased localization in the periphery of the female reproductive tract.

Table 2.1. Linkage of *me69* phenotype to genomic position.

Marker[1]	Genetic position[1]	Genomic position[2]	Haw/+ frequency[3]
WBVar00240399	I:0.91	I:6350803	1/16
WBVar00172772	II:0.12	II:6789208	8/16
WBVar00067953	III:-0.31	III:8318640	10/16
WBVar00188750	IV:1	IV:4625317	3/16
WBVar00240687	V:0.88	V:8177520	9/16

[1]Wicks SR, Yeh RT, Gish WR, Waterston RH, Plasterk RH (2001) Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat Genet* 28(2):160-4. doi:10.1038/88878
[2]Wormbase WS243 (accessed August 30, 2014).
[3]*me69; him-5* males were crossed to CB4856 Hawaiian hermaphrodites, F1 males were crossed back to *me69; him-5* hermaphrodites, and F2 males were assayed for precedence defects in crosses to *spe-8; dpy-4* hermaphrodites. Animals scoring as mutant (*me69* homozygotes) were scored by PCR and restriction digest for centrally-located SNPs on each chromosome (Wormbase release WS243). Animals lacking Hawaiian alleles at all loci tested were considered self progeny and excluded from analysis.

Table 2.2. Mapping of *me69* on chromosome I. F2 males from the cross described in Table 2.1 were scored for SNPs across chromosome I. Animals were either homozygous Bristol (B/B) or heterozygous for the Hawaiian allele (H/B) at each SNP.

No. F2s[1]	WBVar 00240 394[2]	WBVar 00240 397	WBVar 00240 399	WBVar 00155 231	WBVar 00240 416	WBVar 00240 407	WBVar 00159 097	WBVar 00240 414	WBVar 00161 629
	825026	5482531	6351803	8646304	10614690	11472093	12433167	13066381	14154889
16	B/B				B/B				B/B
6	H/B				B/B				B/B
2	H/B	B/B	B/B	B/B	B/B	B/B	B/B	H/B	H/B
3	B/B	B/B	B/B	B/B	B/B	B/B	B/B	H/B	H/B
1	H/B	H/B	H/B	B/B	B/B	B/B	B/B	B/B	B/B

[1]Number of F2 males showing each pattern.
[2]SNP designation and genomic position on chromosome I. Wicks SR, Yeh RT, Gish WR, Waterston RH, Plasterk RH (2001) Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat Genet* 28(2):160-4. doi:10.1038/88878; Wormbase WS243, accessed August 30, 2014.

Table 2.3. Construction of entry plasmids used to generate targeting constructs.

Fragment description	Fragment length	Forward primer	Reverse primer	Vector	Plasmid name
comp-1 promoter	712	GGGACAACTTTGTATAGAAAAGTTGCC AGTTCCTCGCCTAGCTTTC	GGGACTGCTTTTTTGTACAAACTTGAT GCTTTTGATTCGATAGATGATCC	pDONR P4-P1r	pJMH1
comp-1 coding region	1921	GGGGACAAGTTTGTACAAAAAAGCAG GCTCAATGACGTTGGTCGAATCGAAAC	GGGACCACTTTGTACAAGAAAGCTGG GTCTTATTTGCGCTGGAATTGATC	pDONR 221	pJMH2
comp-1 coding region without stop codon	1918	GGGGACAAGTTTGTACAAAAAAGCAG GCTCAATGACGTTGGTCGAATCGAAAC	GGGACCACTTTGTACAAGAAAGCTGG GTATTTGCGCTGGAATTGATC	pDONR 221	pJMH3
comp-1 3' region	561	GGGGACAGCTTTCTTGTACAAAGTGGA AGAACTTACGGAAGAATATG	GGGGACAACTTTGTATAATAAAGTTG ATGCGTTCTCATCAGGCTTC	pDONR P2r-P3	pJMH4
peel-1 coding region[1] without stop codon	3279	GGGGACAAGTTTGTACAAAAAAGCAG GCTGCTTAATGCGCTTTGGTAAG	GGGGACCACTTTGTACAAGAAAGCTG GGTCTGGATTTTCAACACTTGGATC	pDONR 221	pJMH20

[1] Seidel HS, Ailion M, Li J, van Oudenaarden A, Rockman MV, Kruglyak L (2011) A novel sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in *C. elegans*. PLoS Biol 9(7):e1001115. doi: 10.1371/journal.pbio.1001115

Table 2.4. Description of targeting constructs used to generate transgenic worm strains.

Construct	Position 1 pDONR P4-P1r	Position 2 pDONR 221	Position 3 pDONR P2r-P3	Destination Vector	Locus
Pcomp-1::comp-1::comp-1 3' region	pJMH1	pJMH2	pJMH4	pCFJ150[1]	*ttTi5605*
Pcomp-1::H2B::GFP::comp-1 3' region	pJMH1	pCM1.35[2]	pJMH4	pCFJ150	*ttTi5605*
Ppeel-1::comp-1::tbb-2 3' region	*Ppeel-1* [4-1][3]	pJMH2	pCM1.36[2]	pCFJ150	*ttTi5605*
Ppeel-1::comp-1::GFP::unc-54 3' region	*Ppeel-1* [4-1]	pJMH3	pGH50[4]	pCFJ150	*ttTi5605*
Ppeel-1::comp-1::mCherry::unc-54 3' region	*Ppeel-1* [4-1]	pJMH3	*mCherry::unc-54* 3' region[4]	pCFJ150	*ttTi5605*
Ppeel-1::peel-1::GFP::unc-54 3' region	*Ppeel-1* [4-1]	pJMH20	pGH50	pCFJ212[1]	*cxTi10816*

[1] Frøkjaer-Jensen C, Davis MW, Hopkins CE, Newman BJ, Thummel JM, Olesen SP, Grunnet M, Jorgensen EM (2008) Single-copy insertion of transgenes in *Caenorhabditis elegans*. Nat Genet 40(11):1375-83. doi: 10.1038/ng.248

[2] Merritt C, Rasoloson D, Ko D, Seydoux G. (2008) 3' UTRs are the primary regulators of gene expression in the *C. elegans* germline. Curr Biol 18(19):1476-82. doi: 10.1016/j.cub.2008.08.013

[3] Seidel HS, Ailion M, Li J, van Oudenaarden A, Rockman MV, Kruglyak L (2011) A novel sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in *C. elegans*. PLoS Biol 9(7):e1001115. doi: 10.1371/journal.pbio.1001115

[4] Liu Q, Hollopeter G, Jorgensen EM (2009) Graded synaptic transmission at the *Caenorhabditis elegans* neuromuscular junction. Proc Natl Acad Sci USA 106(26):10823-8. doi: 10.1073/pnas.0903570106

CHAPTER 3

POSTCOPULATORY SELECTION IN *CAENORABDHITIS ELEGANS*

Abstract

Sperm competition is the competitive process between two males for fertilization and is thought to be a driving force for rapid molecular evolution and population divergence. The selective pressures stemming from sperm competition have given rise to several adaptions to assist the successful use of male sperm and include sperm number, size and length, and sperm storage. However, females often influence sperm selection through cryptic female choice, but very little is known about the genes or the mechanisms employed in female biased sperm competition.

In *C. elegans,* male sperm compete with hermaphrodite self sperm, resulting in the nearly absolute preferential use of male sperm. Evidence indicates that male precedence relies on intrinsic differences between male and hermaphrodite sperm and strongly implicates disparities in motility. We previously identified a mutant that affects the function of the *comp-1* gene, whose disruption leads to male precedence and migration defects. Our results suggest that the *comp-1* defects are dependent on the competitive context and that the

hermaphrodite reproductive environment likely plays a role in the aberrant mutant sperm migration behaviors. In this study, we have shown that presence of hermaphrodite sperm may have an inhibitory effect on male sperm via a sperm-sensing pathway, involving prostaglandin signaling. Furthermore, this signaling pathway is likely derived from the hermaphrodite in response to the presence of sperm. Our studies show a complex genetic and cellular interaction between male sperm and hermaphrodite reproductive organs. To date, *comp-1* is the only gene identified that specifically affects sperm competition through cryptic hermaphrodite choice. Its characterization likely will reveal a novel mechanism for sperm selection as well as the molecular nature of intercellular communication between sperm and the reproductive tract.

Introduction

Polyandry occurs across many phyla, resulting in sperm competition as a pervasive, postcopulatory method for natural selection (Birkhead and Moller 1998). Sperm competition influences the fertilization success of sperm from different individuals, affecting which individual will contribute his genes to future offspring. Consequently, postcopulatory sexual selection exerts selective pressure on reproductive proteins, resulting in diverse sperm and reproductive tract morphologies (Swanson et al. 2001; Swanson and Vacquier 2002; Eberhard 2004; Clark et al. 2006). These changes in reproductive anatomy and function can have broader evolutionary implications and have been linked to macroevolutionary processes of speciation (Manier et al. 2013).

Postinsemination mechanisms in internally fertilizing species have been notoriously difficult to study and often results are obtained through indirect measurements. While studies of sperm competition have revealed a variety of male and female traits that influence sperm function and usage, many of the molecular and cellular details of these traits remain unknown. Females may also influence the outcome of sperm selection through cryptic female choice. Yet, even less is known about the female's contribution as it is often obscured by male-driven processes. Insight into the molecular pathways regulating sperm competition and cryptic female choice will have a high impact in the field of evolutionary biology as so little is currently known.

Sperm competition occurs when ejaculates from different males compete to fertilize a given set of ova. In order to outcompete another individual's sperm, stored sperm from the first male must resist inactivation or removal. Incoming sperm from a second male must usurp the rival sperm already present. Several traits in males have evolved to increase fertilization success, including production and transfer of numerous smaller sperm in an effort to gain paternity by numerical superiority (Gage 1991). However, in some cases, larger sperm may have an advantage by increasing migration velocity, promoting retention, or blocking subsequent access to the site of fertilization (Wigby and Chapman 2004). Sperm competition can lead to cryptic female choice as females adapt to regain control over which males fertilize their oocytes either through physical or chemical means (Bangham et al. 2003; Lovlie et al. 2013; Lupold et al. 2013). *Drosophila* sperm length is one such example where paternity is biased by the

female favoring sperm with optimal sperm length (Miller and Pitnick 2002). As a result, *Drosophila* sperm length is thought to have coevolved in response to the length of the female's seminal receptacle. For example, *D. bifurca* have sperm approximately 20X the total body length of males (Pitnick et al. 1995b). Ultimately, the fertilization success of sperm is mediated by intrinsic sperm morphology or functions, seminal fluid composition, female reproductive morphology, and intercellular communication between the chemical environment of the female and sperm.

C. elegans is an ideal model system to study the genetic and cellular mechanisms involved in sperm competition and cryptic hermaphrodite (female) choice since the ejaculates of different males can be distinguished and observed within the transparent reproductive tract. *C. elegans* is a male-hermaphrodite species in which hermaphrodites produce their own self sperm which reside in the spermatheca, a sperm storage organ and the site of fertilization (Ward and Carrel 1979). If mating occurs, male sperm migrate through the reproductive tract to the spermathecae, where they encounter stored self sperm. Thus, male sperm must always compete with hermaphrodite sperm; however, male sperm are preferentially used by the hermaphrodite for fertilization. The mechanisms contributing to male sperm's competitive advantage are not well understood, but previous evidence indicates that male precedence relies on intrinsic differences between male and hermaphrodite sperm and requires motility, implicating migration behaviors (LaMunyon and Ward 1994, 1995; Singson et al. 1999).

We identified a gene, *comp-1*, whose loss results in competition-specific

defects in sperm migration, storage, and usage. Additionally, *comp-1* was observed to regulate sperm-intrinsic functions required to compete both offensively and defensively, independent of sperm size. Strikingly, *comp-1* sperm usage defects are specific to competitive contexts, as *comp-1* sperm are functionally normal when wild type sperm are absent (Hansen JM, Chapter 2), suggesting that *comp-1* defects in sperm function may be dependent on changes in the hermaphrodite environment in response to the presence of sperm. Previous evidence supports a model in which sperm communicate with the hermaphrodite reproductive tract to promote induction of intercellular pathways necessary for reproductive processes. *C. elegans* sperm interacts with hermaphrodite environment by two known intercellular signaling mechanisms. Hermaphrodites release prostaglandins, which promote optimal sperm velocity and recruitment to the spermathecae (Kubagawa et al. 2006). In addition, male sperm signal to the hermaphrodite via a paracrine hormone, major sperm protein (MSP), to stimulate oocyte meiotic maturation, gonadal sheath contractions, and ovulation (Miller et al. 2003; Kosinski et al. 2005; Cheng et al. 2008). Sperm also promote egg laying via an unknown signaling mechanism (McGovern et al. 2007). These signals couple costly reproductive processes, such as egg production, to the presence of sperm. To date, the effect of these pathways on sperm competition has not demonstrated.

Our results show that the *comp-1* defects of migration, spermathecal residency, and usage are specific to contexts where wild type sperm are present. Furthermore, the inhibitory affect of wild type sperm is not due to a physical

interaction with *comp-1* sperm, but rather through prostaglandin signaling derived from the hermaphrodite and mediated by the presence of wild type sperm. Specifically, we propose a model in which *comp-1* functions in incoming male sperm to respond favorably to prostaglandin signaling triggered by the presence of previously established sperm. *comp-1* function may have adapted to prostaglandin signaling to either improve motility or to overcome it as an inhibitory signal. Our studies are the first of their kind to reveal the sophisticated molecular interplay between sperm competition and cryptic hermaphrodite choice. Results from our studies of *comp-1* will provide insight into novel mechanisms of sperm competition, cryptic female choice, and coevolution of the sexes.

Results

The presence of wild type sperm inhibit *comp-1* sperm function

Hermaphrodites produce a fixed number of sperm before switching over to oocyte production, resulting in a depletion of self sperm stores as the hermaphrodite ages (Singson 2001). Although *comp-1* male sperm usage increases as the hermaphrodite ages, the levels of *comp-1* male-sired progeny are significantly lower than wild type until a time point that corresponds with a severe reduction in self sperm numbers. *comp-1* sperm have defects in migration, spermathecal storage, and ultimately sperm usage. While *comp-1* male fertility is normal, indicating that sperm function is normal in noncompetitive contexts, it is not known if the migration defect persists when self sperm stores

are low (Hansen JM, Chapter 2). To examine the effect of various amounts of self sperm numbers on *comp-1* migration, we tested the migration of 24 hrs wild type or *comp-1* males mated to hermaphrodite recipients aged 12, 24, 36, or 48 hrs post L-4. At 2 hrs post-mating, the majority of wild type sperm migrate to an area at or near the spermatheca in hermaphrodites aged 12, 24, and 36 hrs, indicating that wild type male sperm migrate rapidly towards the spermatheca immediately after transfer (Figure 3.1A). In a large percentage of crosses with *comp-1* males, *comp-1* sperm had overall significantly less sperm migrate towards the spermatheca than wild type, consistent with the previous result that the sperm usage defect persists in hermaphrodites aged up to 36 hrs. Interestingly, sperm migration in 48 hrs hermaphrodites is conspicuously different. Wild type males mated to a 48 hrs hermaphrodite resulted in only a small percentage of animals with sperm migration rates similar to crosses to a 24 hrs hermaphrodite, suggesting that wild type male sperm do not efficiently migrate towards the spermatheca. However, most crosses with *comp-1* males resulted in the majority of sperm migrating towards the spermatheca within 2 hrs. Although wild type sperm usage increases as the hermaphrodite ages, sperm migration is at least temporally delayed. Ovulation and egg laying rates markedly decrease in older hermaphrodites, suggesting that older hermaphrodites become inefficient at relaying the signal necessary for such processes. Perhaps included in this deterioration is the signal for rapid wild type sperm migration. Surprisingly, *comp-1* sperm lack the delay in sperm migration in older hermaphrodites, in conjunction with the absence of the sperm usage defect coinciding at a time point

in which self sperm stores are depleted and competition between male and self sperm ceases. These results suggest that the presence of wild type self sperm are inhibiting the effective migration and usage of *comp-1* sperm. Since self sperm are localized in the spermatheca, away from the location of transferred male sperm, the inhibitory effect is likely to be due to signaling rather than a physical inhibition of self sperm on *comp-1* male sperm.

To examine the possibility that maternal age contributes to either the decrease in wild type or the increase of *comp-1* male sperm migration rates, we tested migration rates in *fog-2* hermaphrodites, whom fail to produce their own self sperm (Schedl and Kimble 1988). We found that wild type male sperm efficiently migrated towards the spermatheca in *fog-2* hermaphrodites (Figure 3.1B), suggesting that the delay in wild type migration in 48 hrs hermaphrodites may be due to maternal age rather than the absence of sperm. However, the *comp-1* male sperm migration defect was again attenuated in a noncompetitive context, leading us to posit that the presence of wild type self sperm were responsible for the *comp-1* migration defect. Several sperm-sensing pathways have been identified that connect the presence of sperm and reproductive signaling. Signaling induced by the presence of wild type sperm may be detrimental to *comp-1* sperm migration and usage, yet promote wild type sperm migration. However, as the hermaphrodite ages, she may no longer respond to these sperm derived cues, resulting in decreased wild type sperm migration.

Normal migration and usage of *comp-1* male sperm in noncompetitive contexts argues that *comp-1* sperm are able to gain residency in the

spermatheca where fertilization occurs. To directly quantify the number of male sperm occupying the spermatheca, we measured individual male sperm positioning within the hermaphrodite reproductive tract of one focal plane in *fog-2* hermaphrodites. We divided the hermaphrodite reproductive tract into four zones, with zones one through three dividing the uterus. Zone one was most proximal to the vulva while zone three was located just outside of the spermatheca. We mated 24 hrs males carrying a sperm-specific GFP reporter to 24 hrs hermaphrodites and recorded male sperm positioning 6 hrs after mating. Normally after mating, the majority of wild type male sperm reside within the spermatheca or just outside of the spermatheca in zone three (Hansen JM, data not shown). As expected, 6 hrs after mating to the female, wild type sperm were mostly located either in zone three or the spermatheca, with a low percentage of male sperm residing in zones one and two (Figure 3.1C). Strikingly, *comp-1* male sperm positioning was not significantly different from wild type sperm, indicating that *comp-1* defects of migration, spermathecal storage, and sperm usage occur only when sufficient numbers of wild type self sperm are present to inhibit normal *comp-1* behaviors.

Wild type sperm inhibition on *comp-1* sperm is likely due to signaling

After mating, male sperm migrate a relatively long distance from the vulva towards the spermatheca where self sperm are stored. The migration defect of *comp-1* sperm occurs in a location uninhabited by self sperm, suggesting that

previously stored wild type sperm are able to inhibit incoming *comp-1* sperm at a distance. We hypothesized that the presence of sperm hinders *comp-1* sperm by some signaling process that is either directly from sperm or indirectly released from the hermaphrodite as a response to the presence of sperm. *C. elegans* sperm are known to release vesicles that contain the hormone major sperm protein (MSP), that then bind to receptors on oocytes and on the spermathecal sheath (Miller et al. 2003). To test if larger numbers of self sperm, and consequently increased release of MSP, has an effect on *comp-1* migration, spermathecal storage, and usage, we analyzed *comp-1* sperm behaviors in *spe-9* hermaphrodites. SPE-9 is a transmembrane protein found on sperm pseudopod membranes and functions to form the sperm-oocyte interactions necessary for fertilization. *spe-9* sperm have normal motility, MSP signaling, and localization, but do not fertilize the egg (Singson et al. 1998; Hansen JM, data not shown), which leads to the accumulation of larger numbers of self sperm residing at or near the spermatheca. Surprisingly, wild type migration in *spe-9* recipients was severely reduced as compared to their migration in wild type hermaphrodites (Figure 3.2A). Contrary to our expectations, the *comp-1* migration defect was ameliorated in *spe-9* hermaphrodites and migration rates resembled those of wild type males mated to wild type hermaphrodites. The migration rates of wild type and *comp-1* sperm in *spe-9* recipients were reminiscent of those in older hermaphrodites. To further our analysis of *comp-1* behavior, we tested if the spermathecal storage and usage defects were present in *spe-9* recipients. The number of cross progeny sired by *comp-1* males was statistically

indistinguishable from progeny sired by wild type males (Figure 3.2B), indicating that *comp-1* usage is not affected by the presence of *spe-9* self sperm. We might expect that if *comp-1* sperm usage were normal, sperm would need to be present in the spermatheca. We know that *comp-1* sperm are normally present in the spermatheca at lower numbers than wild type male sperm in wild type hermaphrodites, yet they continue to not be used (Hansen JM, Chapter 2). Since *spe-9* sperm are unable to fertilize the oocyte, even smaller numbers of *comp-1* sperm in the spermatheca would outcompete the mutant self sperm. Interestingly, the percentage of *comp-1* male sperm positioned within the spermatheca is similar to that of wild type (Figure 3.2C), implying that *comp-1* sperm are unaffected by *spe-9* self sperm. Together these results suggest that MSP signaling from sperm does not account for the defects in *comp-1* sperm. Therefore, the signal affecting *comp-1* sperm behaviors is from another source, likely released by the hermaphrodite. Sperm may have an additional role involved in promoting an intercellular response from the hermaphrodite, a pathway in which the loss *spe-9* function is unable to fulfill. Previous evidence supports that *C. elegans* sperm has a signaling pathway independent of MSP signaling. *egl-32* hermaphrodites have an egg-laying defect which can be rescued by the presence of wild type sperm, but not by *spe-9* sperm (McGovern et al. 2007). The migration results of wild type sperm in *spe-9* hermaphrodites recipients is consistent with the hypothesis that a signaling pathway exists which promotes wild type sperm migration yet inhibits efficient migration of *comp-1* sperm.

If *egl-32* functions in sperm to induce signaling in the hermaphrodite to promote egg laying, we would expect that *comp-1* male sperm usage potentially might be affected. To test if *comp-1* sperm are efficiently used in *egl-32* hermaphrodites, we tested wild type and *comp-1* male precedence in wild type and *egl-32* hermaphrodites. Notably, we found that both wild type and *comp-1* males mated to *egl-32* hermaphrodites had reduced male precedence as compared to males mated to wild type hermaphrodites (Figure 3.2D). Wild type sperm are able to rescue the loss of *egl-32* function and thus restore the signaling process necessary for egg laying. Despite wild type sperm restoring the signaling pathway for egg laying, they were unable to compete effectively against *egl-32* self sperm. These results provide a condition in which self sperm can outperform male sperm. It is unclear if the increased precedence of *egl-32* self sperm is an intrinsic function or a result of the altered hermaphrodite environment.

Prostaglandin signaling is inhibitory to *comp-1* migration and usage

Several mutant strains have been identified in which loss of function mutations reduce prostaglandin signaling. These mutant hermaphrodite recipients are known to affect sperm migration behaviors including reduced velocity, increased reversal frequencies, and reduced directionality (Edmonds et al. 2010). Since prostaglandin signaling is known to promote wild type sperm migration, it was a likely candidate signaling pathway that differentially affects wild type and *comp-1* migration. To determine the effect of prostaglandin

signaling on *comp-1* sperm migration and usage, we performed the migration and male precedence assay with males crossed to *fat-2*, *fat-3,* or wild type hermaphrodites. *fat-2* and *fat-3* are necessary to synthesize PGF2α prostaglandins and loss of either one is known to reduce prostaglandin signaling (Edmonds et al. 2010). Although the migratory behaviors of wild type male sperm were shown to be altered in *fat-2* and *fat-3* hermaphrodites, self sperm are localized properly in the *fat-2* and *fat-3* hermaphrodites (data not shown). *fat-2* and *fat-3* hermaphrodites have reduced brood size counts, averaging at 85 and 125, respectively (Hansen JM, data not shown). Although self sperm numbers are lower than wild type, there is a sufficient number of self sperm to inhibit incoming *comp-1* sperm according to our previous studies. While wild type sperm are reported to have reduced velocity in *fat-3* hermaphrodites, in most crosses, the majority of wild type sperm reached the spermatheca region within 2 hrs after transfer (Figure 3.3A). Perhaps the reduction in prostaglandins, and not the elimination of the signal, only mildly altered wild type sperm migration. Interestingly, the *comp-1* migration defect was attenuated in *fat-3* hermaphrodite recipients, suggesting that the reduction of prostaglandin signaling is sufficient to restore proper motility to *comp-1* sperm. To test if prostaglandin signaling inhibits *comp-1* sperm usage, we performed the male precedence assay by mating either wild type or *comp-1* males to *fat-2*, *fat-3*, or wild type hermaphrodites. The number of *comp-1* cross progeny was statistically indistinguishable from wild type progeny numbers (Figure 3.3B). The ability of *comp-1* sperm to perform better in *fat-2* or *fat-3* than wild type hermaphrodites suggests that prostaglandin

signaling, while promoting wild type sperm migration, is inhibitory to *comp-1* sperm migration and usage. These data reveal that the response of sperm is more complex than originally thought and may include both stimulatory and inhibitory components. The effect of prostaglandins on cell migration has been the subject of extensive studies, and it is known to both promote and limit cell migration (He et al. 1986), consistent with our observations that indicate prostaglandin signaling can have both a positive and negative effect on sperm motility.

Loss of *vab-1* inhibits *comp-1* in noncompetitive contexts

Loss of *inx-14* and *ceh-18* in hermaphrodites disrupts the function of gap junctions between the sheath cells and oocytes, resulting in reduced velocity and increased reversal rates of male sperm (Kubagawa et al. 2006). Although levels of PGF2α isomer are increased in *inx-14* mutants, *inx-14* is thought to regulate F-series prostaglandin by inhibiting synthesis or promoting catabolism (Edmonds et al. 2011). *inx-14* and *ceh-18* functions are additional downstream components of the prostaglandin signaling pathway. To examine *comp-1* behavior in *inx-14* recipients, we performed male precedence and migration assays. Consistent with previous reports, wild type male sperm were unable to efficiently migrate towards the spermatheca within 2 hrs after mating (Figure 3.4A). Although wild type sperm have similar velocities in *fat-3* and *inx-14*, the reversal rates of wild type male sperm are much higher in *inx-14* than in *fat-3* hermaphrodites (Kubagawa et al. 2006; Edmonds et al. 2011). The increase in reversal rate may account for

the significant reduction of wild type migration rates in *inx-14* hermaphrodite. Migration of *comp-1* male sperm was also impaired in *inx-14;* however, the migration rates were not significantly different from migration rates of *comp-1* male sperm in wild type hermaphrodites, suggesting that *inx-14* recipients have no effect on *comp-1* male sperm migration. Despite slower migration rates of wild type sperm in *inx-14* hermaphrodites, male precedence was normal (Figure 3.4B). Ultimately, the delay in migration of male sperm does not affect its ability to compete, even though it indicates impaired motility. Again, *comp-1* male sperm usage was not affected in *inx-14* or *ceh-18* hermaphrodites (Figure 3.4C), suggesting that *inx-14* and *ceh-18* has no affect on male precedence of wild type or *comp-1* sperm.

MSP signaling to promote oocyte maturation through MAPK activation involves several genes, including *vab-1* in oocytes and *ceh-18* in sheath cells (Miller et al. 2003; Whitten and Miller 2007). Although MSP signaling did not appear to affect *comp-1* function, *vab-1* has roles independent of MSP signaling. To investigate the role of *vab-1* genes in *comp-1* male sperm usage, we assayed male precedence of wild type and *comp-1* males mated to *vab-1* hermaphrodites. *vab-1* hermaphrodites had an average progeny count of 77 at the time of mating, suggesting that sperm numbers were low enough that we would expect to see both wild type and *comp-1* male precedence to increase. While wild type male precedence was high, *comp-1* male precedence remained low even though self sperm numbers were depleted (Figure 3.4D). In order to investigate this result, we analyzed the kinetics of cross and self sperm usage. Even though *comp-1*

male precedence is reduced in wild type hermaphrodites, the total progeny count was the same regardless of if the hermaphrodite was mated to a wild type or *comp-1* male (Figure 3.4E). In the case of the *vab-1* recipient, total progeny counts of the hermaphrodite was reduced when mated to *comp-1* males as compared to wild type males. By comparing the self versus cross progeny production of males mated to *vab-1* hermaphrodites, we see that wild type males sired many cross progeny while there were relatively few self progeny (Figure 3.4F). As expected, *comp-1* males were unable to suppress self progeny production. Yet, even though self sperm numbers were low, the *comp-1* sperm usage defect persisted. This suggests that the *comp-1* sperm are not used even in the absence of competition with self sperm. The loss of *vab-1* in hermaphrodites likely inhibits *comp-1* sperm even in noncompetitive contexts.

comp-1 is involved in the sperm-sensing pathway

Previously we showed that male precedence of both wild type and *comp-1* males increased when mated to *comp-1* hermaphrodites (Hansen JM, Chapter 2). We would expect that wild type sperm would have a higher male precedence since *comp-1* self sperm have difficultly gaining residency within the spermatheca. However, it is less clear why male precedence is reestablished when *comp-1* males are mated to *comp-1* hermaphrodites. Given that *comp-1* sperm function normally in the absence of a sperm-induced signaling pathway, perhaps *comp-1* male sperm gain precedence because they are functioning normally. In order to test this hypothesis, we tested *comp-1* migration in *comp-1*

recipients. We again crossed marked males to wild type or *comp-1* hermaphrodites. Wild type sperm migrated towards the spermatheca of *comp-1* hermaphrodites at rates typical of those established in wild type recipients (Figure 3.5A). Surprisingly, in most crosses, the majority of *comp-1* male sperm migrated towards the spermatheca within the same time frame as that of wild type, suggesting that *comp-1* male sperm migration defect is attenuated in *comp-1* hermaphrodites. This result supports a model in which *comp-1* self sperm are unable to induce the signaling pathway that is inhibitory towards incoming *comp-1* sperm.

Discussion

Our results have shown that previously stored wild type sperm are necessary to trigger the *comp-1* defects of sperm migration, spermathecal residency, and usage. Furthermore, these defects are not caused by the direct interaction between wild type and *comp-1* sperm, but are rather through a long-distance signal. We have shown that in addition to the role of prostaglandin signaling in sperm motility, this signaling pathway can influence *comp-1* sperm behaviors necessary for sperm competition with self sperm. From these results, we propose a model (Figure 3.6) in which previously stored sperm most likely trigger a hermaphrodite-derived signal causing a change in reproductive tract chemical environment. One such signal could be prostaglandins, which promote wild type migration but are detrimental to *comp-1* sperm. *comp-1* may function to respond favorably to prostaglandins in terms of migration speeds and

directionality. Furthermore, *comp-1* may be a necessary component in a sperm intrinsic pathway and its loss perhaps causes additional defects in sperm morphology and ultimately motility. Additionally, *vab-1,* a gene in the MSP sperm-sensing pathway, appears to have a separate function that potentially regulates incoming sperm. Our studies are the first of their type to show that prostaglandin signaling, already known for its role in sperm guidance and reproductive processes, is a mechanism of cryptic hermaphrodite choice through differentially modulating distinct ejaculates. Moreover, our results identified the only known molecular pathway contributing to cryptic hermaphrodite choice.

Absence of sperm and signaling

In *C. elegans*, after mating, male sperm migrate to the site of fertilization where they first encounter self sperm. In order to gain precedence, male sperm must enter the spermatheca, the normal site of fertilization. *C. elegans* male precedence is gained by male sperm predominately occupying the spermatheca and likely by impeding self sperm access to the site of fertilization. Male sperm are so effective at inhibiting self sperm from the spermatheca, that after mating to males with fertilization defective sperm, the hermaphrodite lays unfertilized occytes (Singson et al. 1999). The explanation for *C. elegans* male precedence has predominately been hypothesized to be a consequence of male sperm size, and in other species, larger sperm are more competitive (Gomendio and Roldan 1991; Parker and Begon 1993; Pitnick et al. 1995a; LaMunyon and Ward 1998). Larger sperm size is thought to increase the success of fertilization by increasing

the velocity of sperm and/or blocking the site of fertilization (Gage et al. 2004). However, *comp-1* male sperm dramatically, despite being larger, lose their competitive advantage to self sperm and fail to suppress self sperm progeny, most likely due to an inability to maintain their presence in the spermatheca. Surprisingly, male fertility of *comp-1* males was normal and suggested that *comp-1* defects were dependent on the competitive contexts (Hansen JM, Chapter 2).

Our current results demonstrate that *comp-1* migration was normal when sperm stores begin to decline, a result that coincides with increased *comp-1* sperm usage as self sperm numbers decrease. Additionally, *comp-1* sperm are able to access and maintain their positioning within the spermatheca. Thus, we observed that by all respects *comp-1* sperm retain normal function in non-competitive contexts. Considering that incoming sperm are localized at a relatively long distance away from the spermatheca where self sperm are stored, the inhibitory effect of self sperm is most likely due to a signaling process that can act over a larger distance. Furthermore, our experiments with *spe-9* hermaphrodites indicate that self sperm residing within the spermatheca is not sufficient to block *comp-1* male sperm migration or entry into the spermatheca, consistent with a hypothesis that the source of the inhibitory effect is not the physical presence of self sperm but rather a direct or indirect signal. We hypothesize that the source of the migration and spermathecal residency defects is a transient, intrinsic change within *comp-1* sperm in response to a change in the hermaphrodite environment.

The hermaphrodite oviduct was once thought of as only a conduit joining the uterus and ovaries. However, researchers discovered that the oviduct has a much more dynamic function in murine and porcine organisms since the oviduct responds to the introduction of gametes by actively transcribing atypical genes and producing proteins (Fazeli et al. 2004; Georgiou et al. 2005; Georgiou et al. 2007). While the cellular function of these proteins has yet to be determined, the phenomena highlights the ongoing sperm-oviduct dialogue. In *C. elegans*, intercellular communication between sperm and the hermaphrodite reproductive tract has also been described. Sperm send signals in the form of a hormone, MSP, to promote meiotic maturation, ovulation, and sheath contractions (Miller et al. 2003). Additionally, sperm promote egg laying through an unknown signaling pathway that is independent of MSP signaling (McGovern et al. 2007). The mechanism by which *spe-9* functions has yet to be appreciated; however, loss of *spe-9* does not affect MSP signaling and its concomitant promotion of meiotic maturation (Chatterjee et al. 2005; Kosinski et al. 2005). In an independent pathway, *spe-9* sperm is unable to provide the necessary signal for egg laying (McGovern et al. 2007). The *spe-9* sequence encodes a transmembrane protein containing EGF-like repeats that is localized to the pseudopod of mature sperm. It is thought that *spe-9* might be a ligand to an unidentified receptor on the oocyte (Singson et al. 1998), thus suggesting that *spe-9* may indeed have a role in signaling. EM imaging of sperm within the spermatheca display a close association of the sperm pseudopod with the spermathecal wall, so much so that lipid dyes from the stained male sperm leaches into the wall, consistent with a

hypothesis that sperm have close contacts within the spermatheca (Ward and Carrel 1979). Since SPE-9 is localized to the pseudopod membrane, its loss could also affect its association with the spermathecal wall, raising the possibility that *spe-9* signaling may be mediated by cell-cell contact. Future studies will determine if signaling induced by sperm is dependent on sperm positioning within specific locations within the reproductive tract.

Signaling mediates sperm competition and cryptic female choice

Prostaglandin signaling is known to regulate sperm motility in several organisms, including humans. Although the reduction of prostaglandin signaling affects wild type sperm velocity and directionality, it is not sufficient in stopping the majority of sperm from reaching the spermatheca within a short period time, nor does it inhibit sperm usage (Hansen JM, data not shown). Strikingly, *comp-1* migration and usage is also comparable to wild type levels, indicating that prostaglandin signaling is likely to be at least one of the signals involved in inhibiting *comp-1* function. Although *comp-1* can compete with self sperm in the *fat-3* hermaphrodite, it remains unknown if *comp-1* male sperm can compete with wild type male sperm in a *fat-3* recipient. We would expect that if *comp-1* fully regained its ability to compete and if the hermaphrodite signaling is regulating sperm selection through prostaglandin signaling, *comp-1* should be able to effectively compete with wild type male sperm in the absence of prostaglandin signaling.

Prostaglandin signaling also regulates reproductive processes such as uterine contractions to aid in delivery (Kotani et al. 2000). Although this role for prostaglandin signaling in *C. elegans* is yet to be determined, prostaglandins promote uterine contractions in many vertebrate and invertebrates species. One such species, the field cricket, *Teleogryllus commodus*, has coupled the presence of sperm with the synthesis of prostaglandin signaling for the purpose of oviposition. Virgin female crickets contain high levels of arachidonic acid, a precursor of prostaglandins, which after mating is converted to prostaglandins by a male-derived enzyme sufficient for prostaglandin biosynthesis (Loher 1979; Loher et al. 1981; Stanley-Samuelson and Loher 1983). Future analysis will be needed to demonstrate if prostaglandin signaling occurs as a response to the presence of sperm and if it has additional roles in uterine contractions in *C. elegans*.

If prostaglandin signaling has an essential function for the reproductive health of the hermaphrodite, one might conclude that sperm coopted this signaling pathway to promote their own usage. Although the reproductive tract is known to contribute to sperm selection via cryptic female choice in other organisms, an exciting possibility is that sperm and the reproductive tract co-evolved to mediate this process. We have yet to distinguish if hermaphrodites evolved prostaglandin signaling to select different types of sperm, or if the males responded to the prostaglandin signaling and coevolved a mechanism to enhance their motility in response to the signal.

Several genes that respond to the MSP signaling include *inx-14*, *ceh-18*, and *vab-1*, with VAB-1 identified as a known receptor of MSP on oocytes. *inx-14* and *ceh-18* activity is elicited by MSP binding an unknown receptor on the somatic sheath cells and act in independent, but somewhat redundant, pathways in negatively regulating meiotic maturation and oocyte MAPK activation (Govindan et al. 2006; Whitten and Miller 2007; Govindan et al. 2009). Loss of VAB-1 in adult hermaphrodites does not affect rates of meiotic maturation when MSP is present (Miller et al. 2003; Govindan et al. 2006; Cheng et al. 2008), thus its role in meiotic maturation is thought to be one of modulation. VAB-1 is necessary to downregulate oocyte maturation when hermaphrodites self sperm stores begin to decline which requires the ephrin EFN-2 but not MSP (Miller et al. 2003; Brisbin et al. 2009). We can draw parallels with our own results in which the inhibitory signaling in *vab-1* hermaphrodites is not turned off as self sperm numbers begin to sharply decrease. Our results show that loss of *vab-1* inhibition on *comp-1* sperm usage is independent of *inx-14* and *ceh-18* activity, consistent with *vab-1* having separate functions. Our experiment with *spe-9* revealed that *comp-1* defects could be attenuated in the presence of MSP signaling. Likely, the role of *vab-1* in the *comp-1* defects does not involve MSP signaling. Future studies will be necessary to determine if loss of the VAB-1 ligand, EFN-2, is sufficient to constitutively cause the *comp-1* sperm usage and other defects.

Materials and Methods

C. elegans culture and strains

C. elegans strains were maintained at 20°C, except in the cases where experiments used strains with temperature sensitive alleles. Worms were fed with OP50 *E. coli* bacteria as previously described (Brenner 1974). For experiments necessitating males, *him-5(ok1896)* strains were used as our wild type with *him-5* present in the background of all other male strains (Hodgkin et al. 1979). All strains were derived from the N2 Bristol wild type strain. Other alleles used for experiments were *comp-1(gk1149)* I, *spe-9(hc52ts)* I, *inx-14(ag17)* I, *egl-32(n155ts)* I, *vab-1(dx31)* II, *mIs11[myo-2::GFP, pes-10::GFP and gut::GFP], fat-2(wa17)* IV*, fat-3(wa22)* IV*, dpy-4(e1166) IV*, *j118*[*Pcomp-1::H2B::GFP::comp-1* 3' region] IV, *fog-2(q71) V*, *him-5(ok1896) V,* and *ceh-18(mg17*) X (Singson et al. 1998; Winston et al. 2002; Kosinski et al. 2005; Kubagawa et al. 2006; McGovern et al. 2007; Edmonds et al. 2011; Meneely et al. 2012) and (*C. elegans* Deletion Mutant Consortium, 2012; Hansen JM, unpublished data; Wood and the Community of *C. elegans* Researchers, 1988).

Sperm competition assays and total progeny counts

To test male precedence, we mated either wild type or *comp-1* males marked with a GFP reporter, *mIs11[myo-2::GFP, pes-10::GFP and gut::GFP]*, to *spe-9(hc52ts), fat-2(wa17), fat-3(wa22), egl-32(n155ts), inx-14(ag17), ceh-18(mg57), vab-1(dx31),* or N2 hermaphrodites. For non-temperature-sensitive alleles, we mated 24 hrs post-L4 virgin males to age-matched virgin

hermaphrodites for 24 hrs. Crosses occurred in a 1:1 ratio on plates with freshly seeded lawns. After the 24 hrs period, both males and hermaphrodites were removed and progeny were counted. For the temperature sensitive alleles, *spe-9(hc52ts)* and *egl-32(n155ts),* the L1 hermaphrodites of mutants and controls were shifted to 25°C. At the L4 stage, hermaphrodites were staged and continued being raised for 10 hrs for *spe-9(hc52ts)* or 16 hrs *egl-32(n155ts)* at 25°C before being placed with males at 20°C. In the case of the *spe-9(hc52ts)* male precedence assays, males were 16 hrs post-L4 at the time of mating. Upon reaching adulthood, offspring were scored as either GFP-positive (self) or GFP-negative (cross) progeny and counted. Only plates that contained both GFP-positive and GFP-negative offspring were included in the analysis.

To estimate the number of self sperm remaining in the hermaphrodite reproductive tract at the point in which the experiment began, unmated, aged-matched hermaphrodites were picked in parallel with the male precedence experiments. These hermaphrodites were transferred every 24 hrs until egg laying ceased. All progeny was counted from the time the experiment began.

In the male precedence and progeny counts assays, wild type and mutant animals were tested in parallel to control for variations in experimental conditions. Each experiment was repeated two to four times, unless otherwise noted and representative results are shown.

in vivo sperm migration and localization assays

To assay sperm positioning of male sperm within the hermaphrodite reproductive tract, male sperm was labeled with MitoTracker Red CMXRos (Life Technologies) as in (Stanfield and Villeneuve 2006). All 24 hrs post-L4 marked virgin males were mated to age-matched virgin hermaphrodites on freshly seeded lawns in a 1:1 ratio. Plates were checked every 30 mins for transfer of male sperm or to record sperm positioning. The approximate location of sperm in both arms of the gonad was recorded. Male sperm positioning was recorded for ~2-2.5 hrs after mating. For migration assays using the *spe-9(hc52ts)* hermaphrodites, males and hermaphrodites were treated as in the male precedence assay before mating.

To count individual sperm localization 6 hrs after transfer, virgin 24 hrs post-L4 males carrying the *Pcomp-1::GFP::H2B* transcriptional reporter were mated for 45 mins to age-matched *fog-2(q71)* hermaphrodites anesthetized in a M9 solution with 0.1% tricaine and 0.01% tetramisole (Kirby et al. 1990). For experiments using the temperature-sensitive allele, *spe-9(hc52ts),* males and hermaphrodites were maintained as previously described in the male precedence assay protocol. At 6 hrs post-mating, hermaphrodites, in which mating was successful, were mounted on 2% agar pads and anesthetized with 12mg/mL of levimisole. Images of multiple focal planes of each hermaphrodite were taken. The hermaphrodite reproductive tract was divided into zones as in (Edmonds et al. 2010) and individual male sperm were counted within these zones. The focal plane that had the most sperm in the spermatheca was used for counts.

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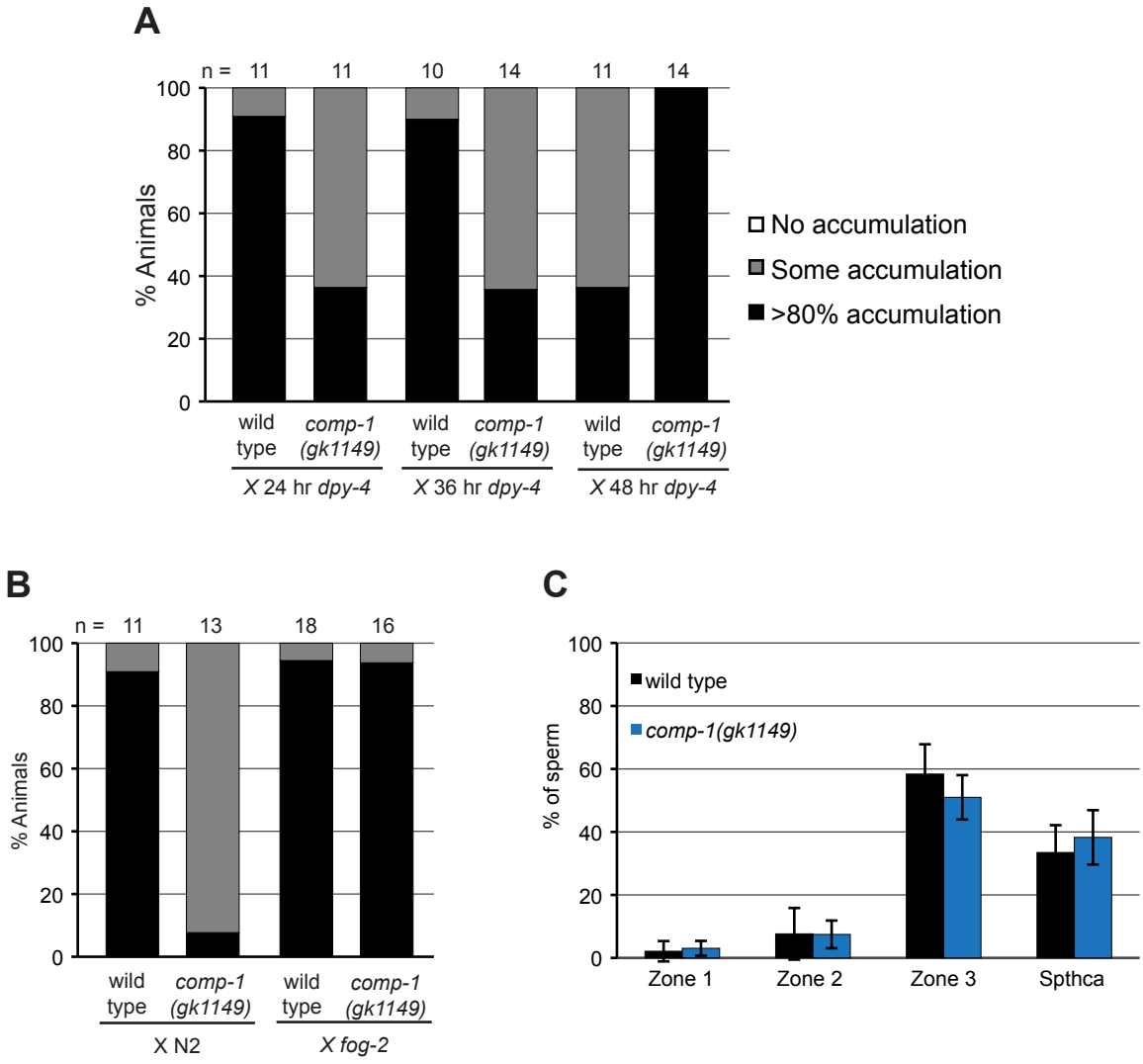

Figure 3.1. *comp-1* sperm function normally in recipients who lack sperm. (A) Wild type male sperm accumulation at the spermathecal region was delayed whereas the *comp-1* male sperm migration defect was mitigated when mated to an older hermaphrodite. Wild type or *comp-1(gk1149)* males stained with Mitotracker were crossed to either *dpy-4* hermaphrodites aged 24, 36, or 48 hrs post-L4. Sperm positioning within the reproductive tract was measured by eye 2-2.5 hrs after sperm transfer and categorized as either more than 80%, some, or no accumulation of sperm near the spermathecal region. n = 10-14 for each genotype with two experimental repeats. (B) *comp-1* migration rates were normal in *fog-2* recipients who lack self sperm. Recipients were *fog-2* hermaphrodites. (C) *jnSi118[GFP::H2B]* sperm localization in 6 hrs after male sperm are transferred to *fog-2* hermaphrodites. Zones are defined in Materials and Methods.

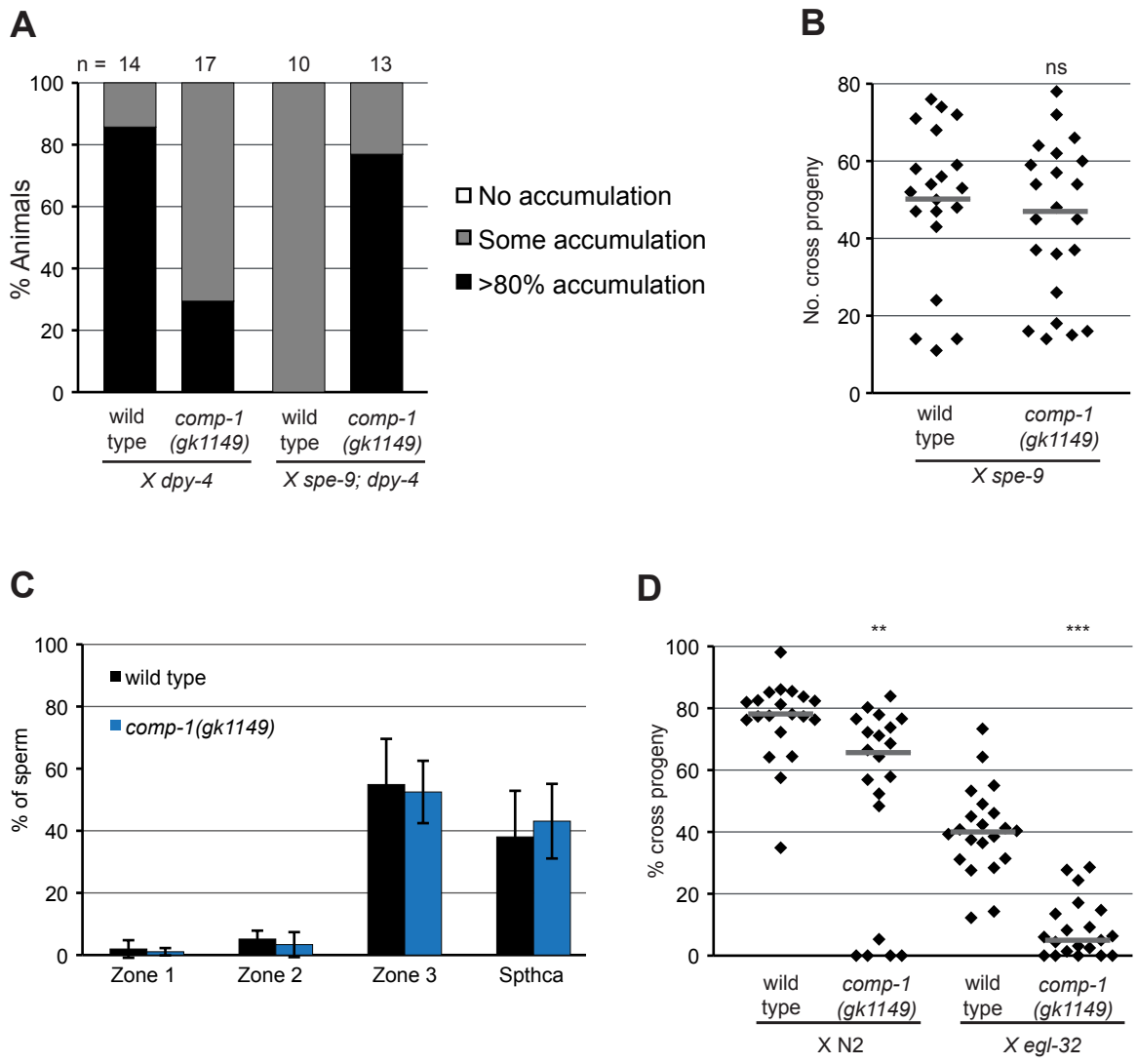

Figure 3.2. *comp-1* function is normal in *spe-9* hermaphrodite recipients. (A,C) *comp-1* migration and spermathecal residency defects were attenuated in *spe-9* hermaphrodites. (B) The number of cross progeny of wild type and *comp-1* was statistically indistinguishable. ns, not significant. (D) Male sperm usage in *egl-32* hermaphrodites was suppressed. Males carrying the transgene *mIS11*(GFP+) were mated to wild type or mutant hermaphrodites and progeny was counted as either cross *mIS11*(GFP+) or self (GFP-). Male precedence is represented as percent cross progeny. Lines indicate medians. *, P<0.05; ***, P<0.001; ns, not significant, Kolmogorov-Smirnov test. Migration and sperm localization assays were performed as in Figure 3.1.

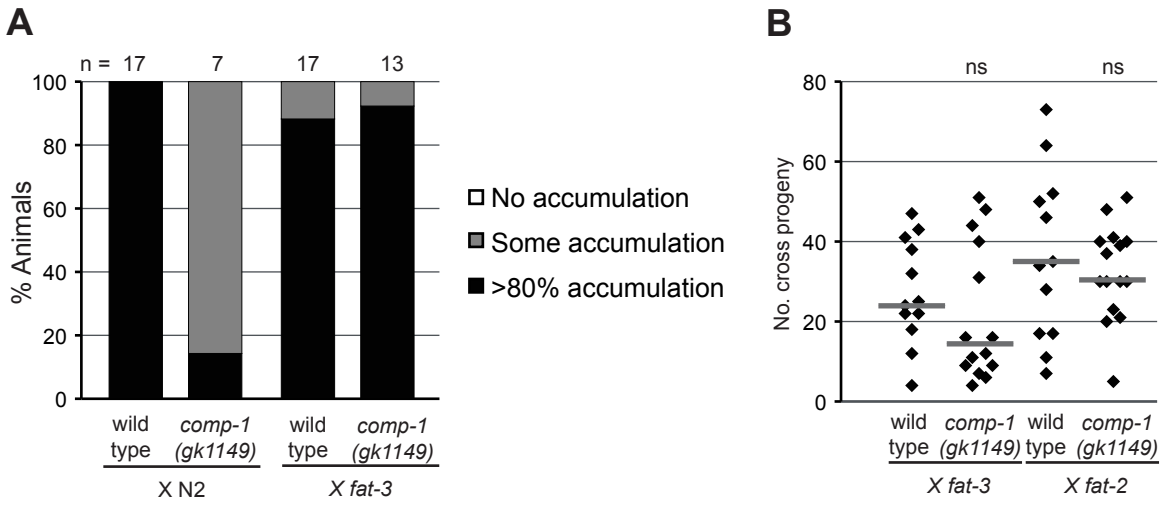

Figure 3.3. Reduced prostaglandin signaling attenuates *comp-1* defects. (A,B) *comp-1* sperm usage and migration was normal in *fat-3* hermaphrodites. Male precedence and migration assays were performed as in Figure 3.1 and Figure 3.2.

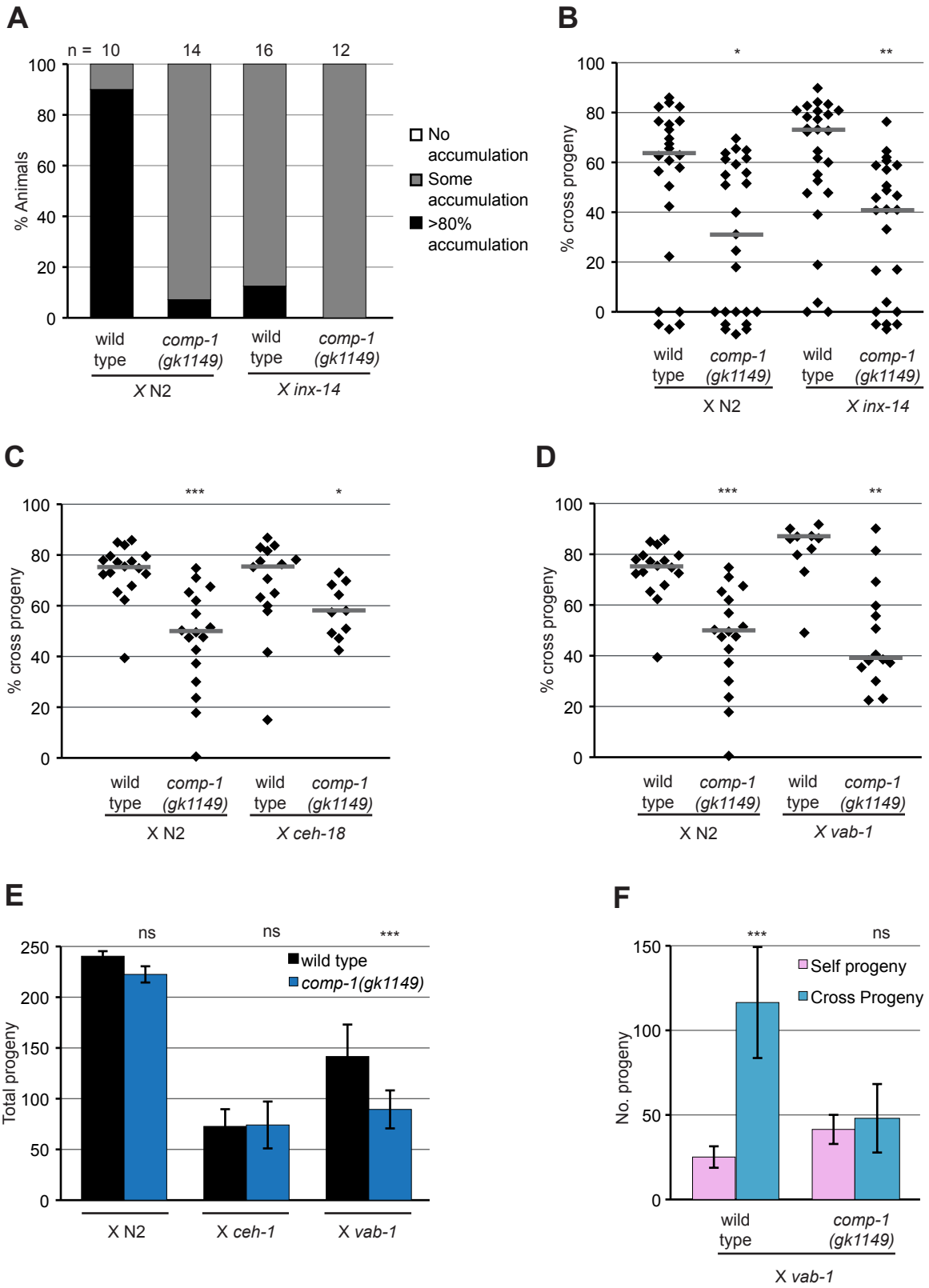

Figure 3.4. Loss of *vab-1* promotes *comp-1* defects. (A,B) The *comp-1* male precedence defect persisted in *inx-14* and *ceh-18* hermaphrodites. (C) Male precedence was reduced in *vab-1* recipients. (D,E)

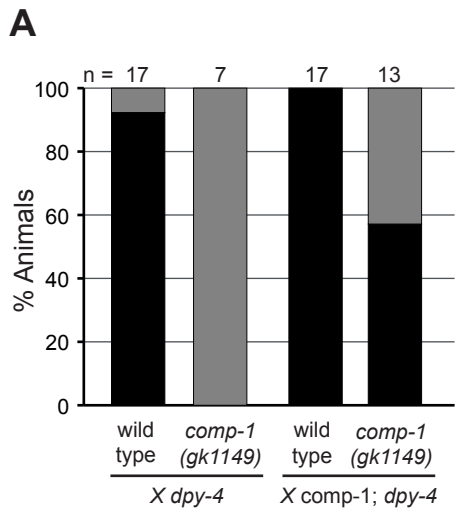

Figure 3.5. *comp-1* is likely to be involved in a sperm-sensing pathway. (A) *comp-1* migration defect was mitigated in *comp-1* hermaphrodites. Migration assays were performed as in Figure 3.1.

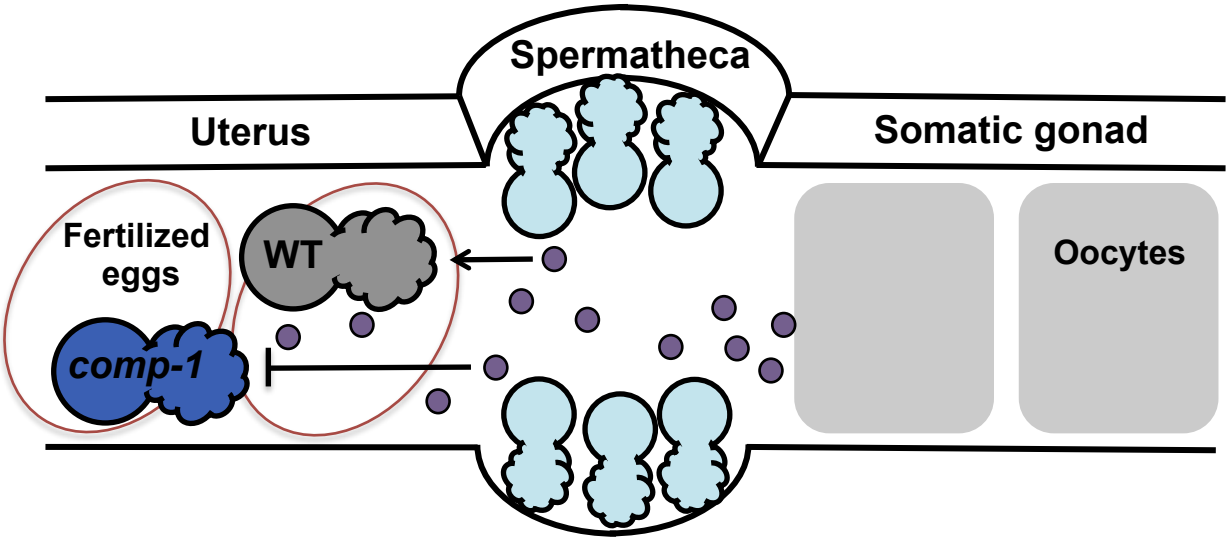

Figure 3.6. Model: *comp-1* is likely to function in a sperm-sensing pathway. The presence of sperm (light blue sperm in the spermatheca) trigger a change in the hermaphrodite reproductive tract. The change is likely to involve prostaglandins (purple circles) which are known to promote wild type sperm migration (grey sperm), whereas *comp-1* sperm (dark blue) are unable to respond to prostaglandin signaling or prostaglandin signaling is detrimental to *comp-1* sperm.

CHAPTER 4

CONCLUSIONS

Summary of Findings

Sperm competition and cryptic female choice exerts selective pressure on reproductive anatomy and protein function, causing rapid divergence of reproductive genes and potentially creating population or species boundaries. Despite sperm competition being widely practiced among many taxa, little is known regarding the direct interaction between sperm and the reproductive tract or between sperm from two different ejaculates. Even less is known about the genes regulating these behaviors. Using a genetic screen, we have identified a gene, *comp-1,* that regulates *C. elegans* sperm competition. *comp-1* regulates sperm-intrinsic functions required to compete both offensively and defensively, independent of sperm size. Furthermore, *comp-1* male sperm display similar defects when competing with wild type male sperm, suggesting that the *comp-1* defects can be extended to male-male sperm competition. Consistent with this idea, *comp-1* is conserved in closely related male-female *Caenorhabditis* species. *comp-1* is the only gene identified that specifically affects *C. elegans* male precedence, and one of few sperm competition genes overall. Its

characterization likely will reveal novel mechanisms for sperm competition and cryptic female choice.

Loss of *comp-1* function results in sperm usage, migration, and storage defects. Strikingly, the *comp*-1 sperm defects are attenuated in hermaphrodites that lack sperm, suggesting that *comp-1* sperm function defects are dependent on the presence of sperm. Specifically, *comp-1* sperm defects in sperm usage, migration, and storage occur only in competitive contexts or when self sperm lack the function to sufficiently signal to the hermaphrodite. Our data suggest a model in which *comp-1* functions in incoming male sperm to overcome inhibitory signals generated in the hermaphrodite reproductive tract triggered by the presence of previously established sperm.

Previous research has established several forms of intercellular communication between sperm and the hermaphrodite reproductive tract necessary for critical events in the reproductive process. In addition, our studies have revealed a sophisticated role for the hermaphrodite reproductive tract in regulating the migratory behaviors of incoming sperm. *comp-1* likely responds to hermaphrodite-derived prostaglandin signaling, a conserved pathway known to regulate many aspects of fertility, to elicit sperm responses such as changes in sperm morphology and migratory behavior that ultimately decide the outcome of competition. Our studies are the first of their kind showing the molecular interplay between sperm and the female/hermaphrodite reproductive tract that regulates competition between sperm.

Evolutionary Consequences of COMP-1

Hermaphroditism arose multiple times in the *Caenorhabditis* genus, which indicates that the ancestral *C. elegans* was most likely a male-female species (Kiontke et al. 2004). *comp-1* is conserved in both male-female and male-hermaphrodite *Caenorhabditis* species. Our results show that *comp-1* is necessary for a competitive advantage in both male-male and male-hermaphrodite competition. Furthermore, it increases sperm usage of male and hermaphrodite sperm in competitive contexts. These results are consistent with the hypothesis that *comp-1* arose in a male-female species and most likely functioned in male-male competition. It is unclear why *comp-1* is so highly conserved in a male-hermaphrodite species since *C. elegans* hermaphrodites mainly use selfing as the primary mechanism to reproduce and males are extremely rare in the population (Hodgkin and Doniach 1997; Barriere and Felix 2005). *comp-1* is dispensable to the hermaphrodite since it does not affect hermaphrodite fertility and *comp-1* hermaphrodites produce as many progeny as wild type hermaphrodites. However, loss of *comp-1* in males is detrimental to their sperm usage when mated to a hermaphrodite. Although outcrossing in *C. elegans* can be rare, there is evidence to support that a mix mating strategy is evolutionary stable (Goodwillie et al. 2005). Certainly, the majority of genes are devoted to male-specific processes (Jiang et al. 2001), and male expressed genes are highly conserved (Cutter 2005), consistent with the hypothesis that male specific genes are being maintained. The rate in which species employ self-fertilization versus crossing varies, suggesting that partial outcrossing can

respond quickly to natural selection (Jain 1976). One explanation of *comp-1* function in *C. elegans* and other species is that it may prevent inbreeding depression, which is particularly important in androdioecious species (Charlesworth and Charlesworth 1987). While 90% of experimental inbred lines of *C. remanei* experienced extinction from inbreeding depression, *C. elegans* outcross lines had reduced fitness as compared to the pure lines resulting in outbreeding depression (Dolgin et al. 2007). The selfing mode of reproduction appears to purge deleterious recessive mutations from the population (Crnokrak and Barrett 2002). Thus, another selective advantage must exist for maintaining males and competitively superior male sperm. One such advantage could be to assure reproductive success in rapidly changing environments and fluctuating populations (Weeks et al. 2006). In populations subjected to novel laboratory maintenance conditions, including changing temperatures, males are present at 5 to 40% of the population (Anderson et al. 2010). Males occur from nullo-X gametes that occur through the rare event of spontaneous nondisjunction. In the laboratory, males can be generated by heat-shock treatment or exposure to ethanol (Sulston and Hodgkin 1988; Lyons and Hecht 1997), suggesting that males are generated when the hermaphrodite is stressed. Indeed, male frequency is much higher in dauer induced populations, where dauer is thought to signify current environmental changes (Barriere and Felix 2005). Therefore, males in general and *comp-1* specifically may serve as a response to promote successful facultative outcrossing due to impending environmental changes.

COMP-1 in Sperm Competition

The role of *comp-1* in sperm competition, and thus sexual selection, is evident by the severe reduction in male precedence. The dramatic reduction in cross progeny production limits the sexual fitness of *comp-1* males and hermaphrodites by markedly diminishing their genetic contribution to future generations. Thus, sperm with *comp-1* function would have a superior competitive advantage to those without its function. Our results support a model in which *comp-1* function gives a strong competitive advantage to male sperm when competing with male or hermaphrodite sperm without *comp-1* function. *comp-1* is necessary for male-male and male-hermaphrodite sperm competition, and thus establishes that male-hermaphrodite sperm competition is an excellent proxy to study male-male competition and to screen for additional genes affecting sperm competition.

To fertilize the oocyte, sperm must migrate to the spermatheca, the site of fertilization. In order to prevent other sperm from fertilizing the oocyte, sperm must either block sperm from another ejaculate from gaining fertilization competency or access to the site of fertilization. *C. elegans* male precedence relies on the principle that after mating, predominately male sperm occupy the spermatheca. After mating to males with fertilization-defective sperm, the hermaphrodite lays unfertilized oocytes (Singson et al. 1999), suggesting that self sperm either did not have access to the spermatheca or were unable to fertilize the oocyte before male sperm. Our results supports that male sperm inhibit self sperm from fertilizing the oocytes since the introduction of male sperm

suppresses self sperm usage. However, *comp-1* male sperm have lost the ability to suppress self sperm progeny production, most likely because male sperm can no longer maintain their position within the spermatheca. Thus, *comp-1* defects in male precedence are likely to be fundamentally due to defects in spermathecal residency. Currently, very little is known about the dynamics of sperm behavior within the spermatheca. Previous observations indicate that the expulsion of the newly fertilized embryo from the spermatheca carrying with it a “cap” of sperm encircling the embryo, necessitating the remigration of sperm back to the spermatheca (Ward 1977). Spermathecal occupancy by male sperm could be governed by two scenarios in which male sperm increase their numbers in the spermatheca by resisting removal and migrating quickly back into the spermatheca after removal by the embryo. EM imaging of sperm within the spermatheca displays a close association of the sperm pseudopod with the spermathecal wall so much so that lipid dyes from the stained male sperm leaches into the wall, consistent with a hypothesis that sperm have close contacts with the wall (Ward and Carrel 1979). Additionally, *C. elegans* sperm are larger in size than self sperm. Theoretical work and previous data suggest that larger sperm size is a direct result of sperm competition (Briskie and Montgomerie 1992; Parker and Begon 1993; Pitnick et al. 1995). Larger sperm size is thought to increase the success of fertilization by increasing the velocity of sperm and/or blocking the site of fertilization (Gage et al. 2004). *C. elegans* male sperm are larger than self sperm; however, how size influences male precedence in *C. elegans* is yet to be resolved but may act in a manner similar to sperm of

other organisms by blocking access or increasing migration speeds to the sperm storage organ. Our results suggest that size alone is not sufficient to determine male precedence, since loss of *comp-1* overrides any influence that size may have. Interestingly, *comp-1* male sperm are able to regain male precedence when competing with *comp-1* self sperm. Several factors are thought to promote male precedence, such as larger sperm size and faster crawling speeds. Loss of *comp-1* in both sexes may cause male sperm to rely on other sperm behaviors to increase their competitive advantage, such as size. The necessary experiment to determine if size is that factor is to compete *comp-1* male sperm of different sizes against each other. Since *comp-1* has defects in migration, *comp-1* sperm may not be able to easily gain initial access to the spermatheca or are unable to re-migrate after removal by the embryo. Alternatively, *comp-1* sperm may have an additional defect that renders them unable to adhere to the spermathecal wall, allowing wild type sperm to preferentially bind and displace *comp-1* sperm. Future studies will be necessary to differentiate behaviors between wild type and *comp-1* sperm, specifically within the spermatheca. These studies will determine if *comp-1* sperm can enter the spermatheca, maintain their position by adhering to the spermathecal wall, and quickly remigrate back to the spermatheca when removed. Results will discern between different mechanisms that wild type sperm may use to gain a competitive advantage over *comp-1* sperm.

Wild type sperm development culminates in the maturation of motile sperm competent to crawl with the use of its fully extended pseudopod, essential for motility and fertilization (Ward et al. 1981). We reasoned that the reduced

aspect ratio of *comp-1* sperm directly produces the defects in migration and spermathecal residency. The short pseudopod length would certainly lead to reduced velocities as well as inhibiting *comp-1* sperm from projecting its pseudopod into the spermathecal wall. Previous observations revealed that fertilization-defective sperm with shortened pseudopods did not associate with the spermathecal wall and were relegated to the lumen of the spermatheca, where they were quickly washed out by the movement of embryos (Ward et al. 1981). Strikingly, defects in aspect ratio were also context-dependent, raising the question of whether the *in vitro* defects coincide with the *in vivo* defects. Such a possibility would suggest that the chemical environment of the reproductive tract, influenced by the presence of sperm, regulates sperm morphology.

Another remaining question is the cellular basis that is the cause of the *comp-1* sperm defects. Interestingly, *comp-1* sperm have condition dependent defects in aspect ratio; specifically, *comp-1* sperm have shorten, misshapened pseudopods. *C. elegans* sperm morphology, as well as other migrating cells, can be modulated by several factors, such as mislocalization of proteins or the misregulation of cytoskeletal components, such as Major Sperm Protein (MSP) in nematodes (Sepsenwol et al. 1989). However, after examination of several sperm structures in *comp-1* sperm, sperm proteins were properly localized. Thus, it is more likely that the defects in *comp-1* sperm morphology stem from the failure to fully extend its pseudopod. However, it is not clear if addition of the weak base, TEA, provides a hospitable environment or if Pronase induces the

defects. Intracellular pH is a known regulator of MSP assembly and disassembly, with a higher pH found at the leading edge where new filaments assemble (King et al. 1994). In wild type sperm, decreasing the pH *in vitro* causes MSP assembly to slow or halt without affecting the disassembly at the base of the cell (Italiano et al. 1999). Since MSP filaments can assemble in a wide range of pH levels, it is thought that differences in intracellular pH alters the activity of membrane proteins that directly affect MSP assembly (King et al. 1992). The increase in pH by the addition of TEA may restore the intracellular pH gradient of *comp-1* sperm. If so, *comp-1* may function to regulate the intracellular pH of sperm. Differences in ovarian fluid pH is known to enhance sperm velocity in rainbow trout (Wojczak et al. 2007), indicating that pH is known to affect sperm behaviors associated with sperm competition. To test if pH regulates *comp-1* morphology, an experiment in which modifying the pH of the sperm buffer could reveal differences in aspect ratio. The results will determine if pH is the factor causing the shortened aspect ratios and may also suggest that *comp-1* sperm have a narrow range of tolerance to environmental pH. More importantly, future investigations into the morphology of sperm *in vivo* will determine if this phenomena is biologically important in the hermaphrodite reproductive tract.

COMP-1 in Cryptic Hermaphrodite Choice

Interestingly, *comp-1* male sperm had normal fertility when self sperm were not present. Further investigations revealed that *comp-1* sperm migrate to and accumulate normally within the spermatheca as self sperm stores are

depleted. Together, these results suggest that the presence of wild type sperm alters *comp-1* sperm behaviors. One mechanism of sperm competition in other organisms involves sperm directly displacing sperm from another ejaculate from the reproductive tract. However, if wild type sperm were inhibiting *comp-1* migration, it would suggest that wild type sperm are suppressing *comp-1* sperm migration at long distances perhaps by altering the chemical environment of the reproductive tract. *comp-1* had normal migration in the fertilization defective *spe-9* hermaphrodites, suggesting that the physical presence of sperm themselves is not the barrier to *comp-1* migration and spermathecal residency. It is known that *spe-9* is necessary for sperm-egg interactions (Singson et al. 1998); however, the cellular mechanism of *spe-9* function has not been determined. SPE-9 is a transmembrane protein consisting of several EGF-like repeats, a conserved domain known to be both a ligand for signaling and involved in cell adhesion (Hynes and Zhao 2000; Swindle et al. 2001). From studies suggesting that *spe-9* may function in intercellular communication between the sperm and the hermaphrodite (McGovern et al. 2007), we hypothesize that *spe-9* sperm may lack competency for crucial signaling processes such as the signals affecting *comp-1* sperm. Again, since *spe-9* sperm positioned in the spermatheca lost their inhibitory effect on incoming *comp-1* sperm migration, *spe-9* most likely plays a role in signaling. However, cell adhesion is also a cellular process known to be very important for sperm storage and competition in other organisms and we cannot rule out that sperm adhesion may be another aspect in which *spe-9* fails to effectively compete with *comp-1* sperm for positioning within the spermatheca.

An alternative hypothesis is that it is the act of fertilization itself that triggers the signal that is so harmful to *comp-1* function. Future studies with other fertilization-defective mutants will be necessary to determine if this is specific to *spe-9*, or if the general process of fertilization is to blame for the *comp-1* defects.

Perhaps the most exciting result from studies with *comp-1* was that the *comp-1* defects in migration and usage were ameliorated in hermaphrodites with reduced prostaglandin signaling. Prostaglandins secreted by the hermaphrodite are known to be important for proper wild type sperm velocity and directionality (Kubagawa et al. 2006; Edmonds et al. 2010). Our studies have shown that prostaglandins are inhibitory to *comp-1* function, suggesting that the hermaphrodite environment directly regulates sperm behaviors and function. These results are the first to establish a specific molecular pathway involved in cryptic hermaphrodite choice. Additionally, it provides evidence for a novel mechanism of how females may bias sperm selection. We hypothesize that the presence of wild type sperm induces prostaglandin signaling which promotes wild type sperm migration yet hinders *comp-1* sperm. *comp-1* may be necessary to favorably respond to prostaglandins by promoting cell processes involved in motility. To test this model, we will need to show that prostaglandins secreted by the hermaphrodite are the cause of the *comp-1* defects. Our experiments did not rule out that the prostaglandin mutant recipients may have impaired self sperm development and therefore, the self sperm were not as competitive. We can approach this question in multiple ways, one of which is to inject prostaglandins into a *fog-2* female to induce the *comp-1* defects or to set up male-male

competition in the prostaglandin mutant hermaphrodite environment so that wild type male sperm are competing with *comp-1* male sperm. Additionally, to investigate cellular responses to prostaglandins, *in vitro* experiments can be used to determine if prostaglandins affect *comp-1* sperm morphology and velocity. It will be absolutely necessary to determine if the presence of sperm is triggering the release of prostaglandins. There is some evidence that the molecular pathway regulating prostaglandin signaling involves genes that respond to MSP signaling (Edmonds et al. 2011); however, these genes to do not appear to affect *comp-1* male precedence. We expect clarification on this result as the research group involved in studying the prostaglandin signaling in *C. elegans* continues their exciting studies on the pathways necessary for prostaglandin signaling.

Our prostaglandin results provide a link to the actual biochemical change in hermaphrodites causing the context-dependent *comp-1* defects. Another interesting result from our studies is the persistence of the *comp-1* defects in ephrin receptor, *vab-1,* mutant hermaphrodites, despite the fact that these hermaphrodites may lack enough sperm to promote these defects. We will need to first confirm that loss of *vab-1* promotes *comp-1* defects by repeating our migration, sperm usage, and spermathecal residency assays in hermaphrodite recipients that completely lack sperm. *vab-1* is known to function in hermaphrodite reproductive processes in response to MSP signaling (Miller et al. 2001; Miller et al. 2003). Yet, in our model, MSP is not the sperm signal responsible for the changes in hermaphrodite reproductive tract that are so inhospitable to *comp-1* sperm. *vab-1* has several MSP independent functions

which includes downregulating oocyte maturation as self sperm run out discussed in (Han et al. 2010). Careful studies will be needed to tease out the role of *vab-1* in *comp-1* function.

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