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Sperm precedence in a hermaphroditic Nematode (*Caenorhabditis elegans*) is due to competitive superiority of male sperm

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Abstract. When male and hermaphrodite *Caenorhabditis elegans* mate, the male's sperm outcompete the hermaphrodite's own sperm and fertilize a majority of the offspring. Here, we investigate the mechanism of male sperm precedence. We rule out the possibility that male sperm are stronger and more competitive because they are activated later than hermaphrodite sperm. We also find that a previously known gender difference in sperm activation does not influence sperm competition. Male sperm, rinsed free of seminal fluid, retained the capacity to take precedence after artificial insemination. Therefore, we conclude that male sperm themselves are competitively superior to hermaphrodite sperm. This trait maximizes outcrossing after mating and may increase both genetic diversity and heterozygosity of offspring whose parents, due to self-fertilization, may be highly homozygous.
Key words. *Caenorhabditis elegans*; mating; sperm competition; sperm activation; sexual dimorphism; outcrossing.

The hermaphroditic, bacteriophagous nematode *Caenorhabditis elegans* is an established model organism in developmental biology, genetics and neurobiology. Only recently has this worm become the subject of ecological and evolutionary studies that capitalize on the availability of mutants and the depth of knowledge accumulated on *C. elegans*[1,2]. Here, we examine the mechanism of sperm competition in *C. elegans* and discuss its evolutionary implications.

In *C. elegans*, reproduction occurs primarily through self-fertilization. The hermaphrodite's bilobed reproductive tract initially generates approximately 300 spherical spermatids. These are activated by a hermaphrodite-specific factor to become amoeboid spermatozoa[3] capable of fertilization, and they reside clinging to the walls of the two spermathecae[4]. Oogenesis follows, and the oocytes are fertilized when they contact the sperm as they pass through the spermathecae. After exhausting their supply of sperm, hermaphrodites lay unfertilized oocytes. Self-fertilization in *C. elegans* allows individual worms to colonize new environments without the need to mate, but as with other forms of inbreeding, it may also result in reduced genetic variability[5].

In addition to self-fertilization, mating occurs between male and hermaphrodite *C. elegans*. Unmated hermaphrodites produce male progeny through infrequent non-disjunction of the sex chromosome (males: XO; hermaphrodites: XX[6]). Males mate readily with hermaphrodites and ejaculate spermatids that are activated in the hermaphrodite uterus by an activating factor passed in the seminal fluid[3]. The male spermatozoa crawl to the spermathecae and subsequently fertilize nearly 100% of the progeny[4]. Thus, outcrossing is maximized by male sperm precedence, yet the mechanism by which the male sperm displace the hermaphrodite sperm is unknown.

One possible mechanism that has already been ruled out is that copulation stimulates hermaphrodites to incapacitate their own sperm. When hermaphrodites copulate with mutant males that pass no sperm, fertility is not reduced[7]. Also, male sperm precedence is not likely due to male sperm outnumbering hermaphrodite sperm (numerical sperm competition). It was observed earlier that male sperm actively displace hermaphrodite sperm from the walls of the spermatheca and take up residence in prime position for fertilization[4], indicating a physical inequality between sperm types. A numerical sperm competition model predicts that all sperm have an equal chance of fertilization; the fraction of offspring fertilized by male sperm should be constant and reflect their numerical representation in the spermatheca. Instead, male sperm fertilize eggs in a burst soon after mating and typically become depleted thereafter, again indicating male sperm superiority[4].

Here we test four hypotheses that could explain male sperm precedence. (1) Hermaphrodite sperm are less vigorous than male sperm because they become active spermatozoa much earlier and thus may deplete limited resources that are essential for sperm competition. From this resource depletion hypothesis, we predict that if hermaphrodite sperm were activated at the same time as male sperm, no displacement should occur. (2) Activation by the male activator endows sperm with a competitive advantage[3]. According to this hypothesis, hermaphrodite sperm activated by the male factor should not be displaced by male sperm. (3) Male seminal fluid incapacitates hermaphrodite sperm. If seminal

fluid determines sperm competition, male sperm should not displace hermaphrodite sperm in the absence of seminal fluid. (4) Male sperm cells are competitively superior to hermaphrodite sperm cells. We predict that male sperm should displace hermaphrodite sperm in the absence of seminal fluid, and hypotheses (1) and (2) above should be rejected. The results of our experiments support the hypothesis that male sperm are competitively superior to hermaphrodite sperm, regardless of the interval between activation and competition, the mode of activation, or the presence of seminal fluid.

Materials and methods

To promote mating between worms for our sperm competition experiments, we confined hermaphrodites and males in petri plates containing agar seeded with *Escherichia coli* strain OP50 as a food source for the worms[8]. Hermaphrodites at the last larval stage (L4) were each paired with 4 young adult males. Worms used in mating experiments were either wild type (Bristol, strain N2) or homozygous for the spermatogenesis defective mutation *spe-8(hc53)* alone or in combination with the morphological marker *dpy-5(e61)*. Generally, the mating interval lasted 4–15 h, during which the hermaphrodites became adults and were mated multiply by the males. A long mating interval was necessary to ensure sufficient insemination because many copulations result in no sperm transfer, and even when sperm are successfully transferred, they may be expelled after mating[1,4]. Thus, it is impossible to count accurately the number of sperm transferred during mating. By allowing the hermaphrodites to mature in the presence of males, we ensured that hermaphrodites were mated as early as possible and thus had an undepleted complement of self sperm. After mating, the hermaphrodites were transferred to fresh plates at regular intervals (generally 24 h or less) until they died or began to lay unfertilized oocytes. After the eggs hatched, the emergent larvae were maintained on plates until they grew to at least the last larval stage, when their paternity was assessed.

Paternity was assigned directly for offspring of *dpy-5* hermaphrodites since these worms are shorter and fatter than wild type, and their self fertilized progeny are easily distinguished from outcross progeny by their dumpy phenotype. Alternatively, in crosses between worms with wild type morphology, the paternity of the offspring was estimated by gender. Because half of the male sperm carry no X chromosome, half of the outcross progeny on average are male. We thus estimated total outcross progeny by doubling the number of male offspring to account for hermaphrodite progeny sired by the male. In cases where male progeny outnumbered hermaphrodite progeny, all offspring were scored as outcross.

We tested the effect of seminal fluid on sperm competition by artificially inseminating hermaphrodites[9] with male spermatids washed by two methods. Our first method entailed rinsing male spermatids within the insemination needles. Spermatids were drawn from the seminal vesicles of wild type males into needles that had been pre-loaded with sperm medium (SM)[10]. More SM was drawn up through the tips of the needles, rinsing the spermatids well up into the needles. The needles were then centrifuged at approximately $200 \times g$ to reconcentrate the spermatids in the tips of the needles. These diluted and reconcentrated spermatids were then injected through the vulva of young-adult *dpy-11(e224)* hermaphrodites. As with the *dpy-5* mutation, self-fertilized progeny of *dpy-11* hermaphrodites are distinguished from outcross progeny by their dumpy phenotype.

We also washed male spermatids during bulk sperm isolation, a method that gives much greater dilution of male sperm than does needle washing. We raised mass cultures of the mutant *him-5(e1490)*, which has a high proportion of male progeny through increased non-disjunction of the sex chromosome. Males were collected and separated by established procedures[11]. By this technique, we obtained approximately 2×10^5 males. To collect spermatids, we mass dissected the males by dicing with a razor for 10 min under a modified SM buffer (45 mM choline Cl, 25 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, 10 mg/ml bovine serum albumin, pH 7). The buffer also contained protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin A) to prevent premature sperm activation through proteolysis. The worm remains were poured over a 10 μm Nitex filter to separate spermatids (diameter 5–6 μm) from other cellular debris[11]. The filtrate was layered on a cushion of 50% Percoll (Sigma Co.) in modified SM and centrifuged at approximately $7{,}000 \times g$ for 3 min. The spermatids were collected from atop the cushion, transferred to SM, and pelleted at $1{,}000 \times g$ for 3 min. The supernatant was removed and the spermatids resuspended in more SM, and the cells were pelleted again. Most of the supernatant was removed, and the spermatids were resuspended in the remaining SM. The spermatid suspension was loaded into an insemination needle, and the needle was centrifuged to concentrate the spermatids in the tip of the needle. These thoroughly washed spermatids were then injected into *dpy-11(e224)* hermaphrodites.

Results

By 10 h after mating between wild type worms, the male sperm fertilized almost 100% of the eggs (fig. 1). Some hermaphrodites produced no subsequent self-fertilized progeny. However, beginning several days after the mating interval, most hermaphrodites laid a decreasing

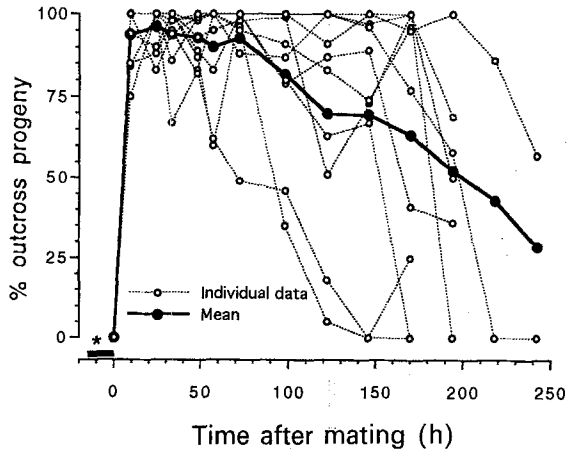

Figure 1. The percent of progeny fertilized by male sperm (outcross progeny) as a function of time after wild type (N2) hermaphrodites mated with N2 males. *Mating interval.

proportion of male-sired eggs (fig. 1), presumably as their supply of male sperm became exhausted. These hermaphrodites produced an average of 695 male-sired progeny (SEM: 47) and 50 (SEM: 16) self-fertilized offspring, but the selfed progeny generally appeared near the end of the reproductive period. Thus, the male sperm did not fertilize a constant fraction of offspring as predicted by numerical competition; they fertilized a disproportionately large share of the eggs immediately after mating. An alternative explanation for the disproportionate fertilization pattern is that male sperm become inviable several days after insemination. However, the mated hermaphrodites that produced mainly male-sired offspring for their entire lives provide evidence that male sperm do not suffer from decreased viability.

Resource depletion hypothesis (1). According to this hypothesis, male sperm should not take precedence if they are activated at the same time as hermaphrodite sperm, because neither sperm would have depleted resources. Results of our experiments with *spe-8* worms contradict the prediction. Hermaphrodite *spe-8* mutants are self sterile because their sperm fail to become activated by the hermaphrodite activator; if *spe-8* hermaphrodites mate, however, their sperm activate by a factor in the ejaculate that normally activates the male sperm after mating[3]. Thus, when *spe-8* hermaphrodites were mated by males in our experiments, both sperm types activated at the same time and by the same activator. However, the male sperm still took precedence over *spe-8* hermaphrodite sperm as they do in wild type hermaphrodites (fig. 2).

In this experiment with *spe-8* hermaphrodites, the potentially positive effect of sperm coactivation was not countered by a general negative effect of the *spe-8* mutation. Sperm from *spe-8* males carry the same defect as *spe-8* hermaphrodite sperm[3] and therefore, should experience the same negative effect; however, even they took precedence over *spe-8* hermaphrodite

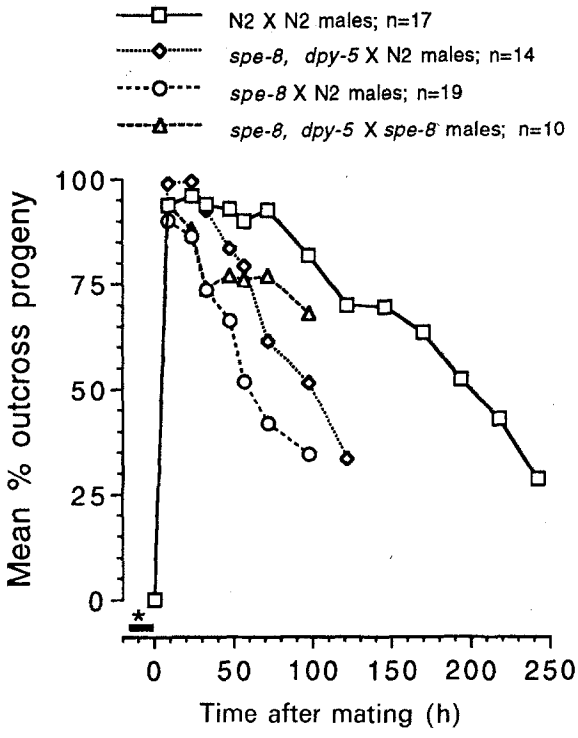

Figure 2. Outcross progeny as a function of time after *spe-8* hermaphrodites mated with N2 or *spe-8* males. *spe-8* hermaphrodite sperm do not activate unless the hermaphrodite mates; both sperm activated simultaneously. N2 × N2 data from figure 1 included for comparison. *Mating interval.

sperm (fig. 2). Thus, our results with *spe-8* were not due to pleiotropic effects of the mutation. Also, the *spe-8* hermaphrodite sperm did not lose out simply because they were too few to compete effectively; when mated to *fer-1(hc13)* males (which are sterile themselves but activate *spe-8* self sperm), *spe-8* hermaphrodites produced on average 215 self-fertilized progeny (SEM = 10, n = 10). Thus, *spe-8* hermaphrodites have near wild type numbers of self sperm (300). Therefore, we reject the resource depletion hypothesis.

Mode of activation hypothesis (2). Results from *spe-8* matings contradict the prediction that hermaphrodite sperm activated by the male factor should be as competitive as male sperm. Even though the sperm of *spe-8* hermaphrodites became male-activated during matings with fertile males, they were subsequently displaced by the male sperm (fig. 2). However, because the male sperm were passed with the seminal fluid, they were probably the first to become activated. Normally, the hermaphrodite sperm activate first and become ensconced in the spermathecae before mating. We subjected *spe-8* hermaphrodite sperm to a more natural course of events by pairing the hermaphrodites first with sterile *fer-1* males for 15 h. This allowed the hermaphrodite sperm to activate in male seminal fluid prior to sperm competition. During a 3-h period after the *fer-1* mating, approximately two thirds of the hermaphrodites laid eggs. These worms whose self

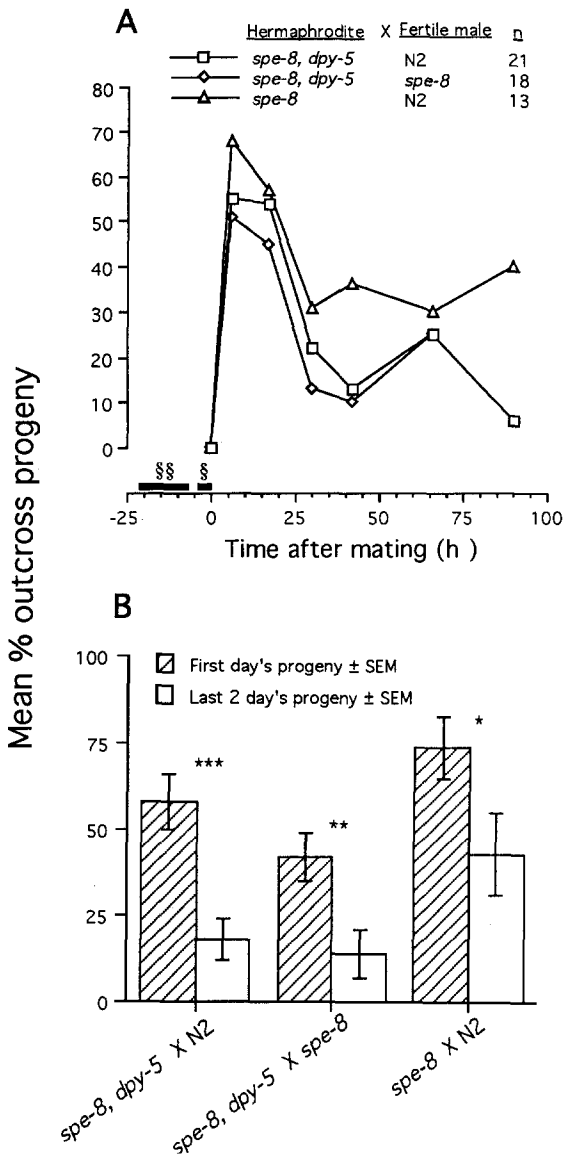

Figure 3. *A*) Outcross progeny as a function of time after *spe-8* hermaphrodites mated with fertile males. The hermaphrodites' sperm had become male-activated through an earlier mating with sterile *fer-1* males. § Fertile male mating interval. §§ Sterile male mating interval. *B*) The outcross progeny from the first day after mating and the last 2 days of egg laying for the *spe-8* hermaphrodite data in the upper panel. (Hermaphrodites laid eggs for 4 days on average.) Data compared statistically by paired t-test after arcsine transformation[33]. p-values adjusted for simultaneous tests by sequential Bonferroni method[34]. ***$p < 0.00001$, **$p = 0.022$, *$p = 0.039$.

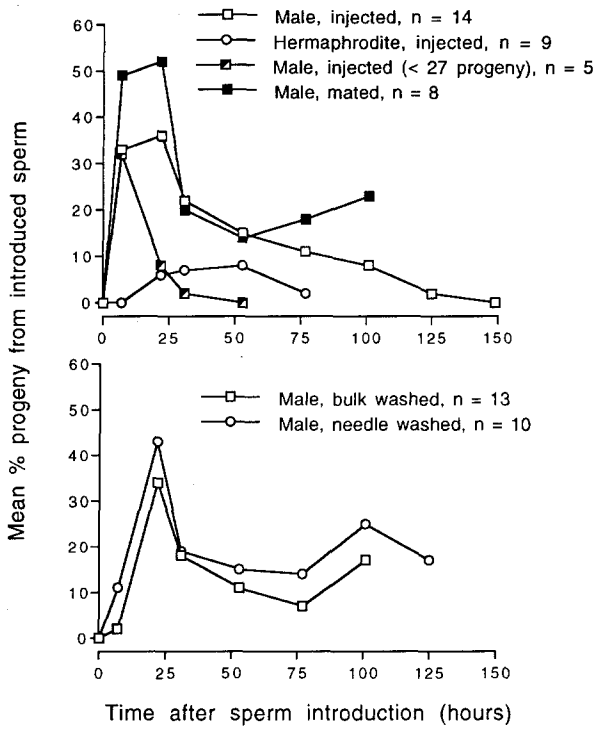

Figure 4. Progeny from sperm introduced into *dpy-11* hermaphrodites by mating or artificial insemination as a function of time after introduction. *Upper panel.* Sperm injected directly after removal from either wild type males or wild type hermaphrodites. Included for comparison are data from mated *dpy-11* hermaphrodites (paired as L4 larvae with wild type males for 4 h). *Lower panel.* Sperm injected after it was washed (see text for details).

sperm had been activated were then paired with fertile males (either wild type or *spe-8*) for 4 h. Our manipulations had no effect on sperm competition: the male sperm still took precedence (fig. 3). While it did not reach nearly 100% as in previous experiments, the share of progeny fertilized by the male sperm dropped significantly after the initial peak at 50% to 70% (fig. 3A and B; see legend for details of statistical analysis). Thus, the male sperm fertilized oocytes preferentially soon after mating; precedence was not complete probably because the mating interval was relatively short (4 h instead of 15 h as in the previous experiments) resulting in fewer matings and less sperm transferred.

Seminal fluid/sperm cell hypotheses (3), (4). Artificial insemination of washed male sperm provided the test for these two hypotheses. The washed sperm took precedence over hermaphrodite sperm in a pattern similar to that observed for unwashed, artificially-inseminated male sperm and for naturally-inseminated sperm (fig. 4). Although the bulk-isolated sperm were diluted to a much greater extent than were the needle-washed sperm (three rinses to one), both methods gave similar patterns of male sperm precedence (fig. 4B). If seminal fluid were responsible for male sperm precedence but was not completely removed by washing, the two methods should have given different results based on the extent of dilution, which they did not. Thus, while we cannot rule out the possibility that dilute seminal fluid was present, even in the bulk-isolated sperm, we reject the hypothesis that it promotes male sperm displacement.

As we found for offspring of mated worms (fig. 3), the fraction of progeny fertilized during the first day after injection was significantly greater than the proportion fertilized during the last two days of egg laying for the bulk-washed sperm and approached significance for the

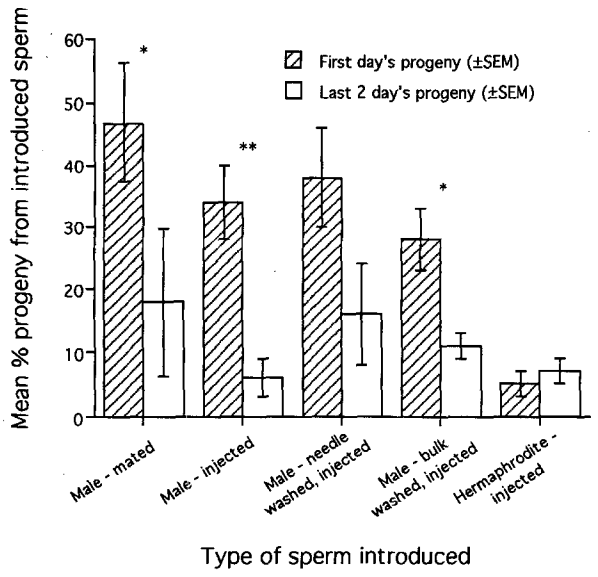

Figure 5. The percent outcross progeny from the first day after mating and the last 2 days of egg laying for the data from artificially inseminations presented in figure 4. (Hermaphrodites laid eggs for 4 days on average.) Statistical treatment of data as described in figure 3 legend. $^{**}p \leq 0.001$, $^{*}p \leq 0.02$.

needle-washed sperm (fig. 5). Because of problems with dust particles clogging the insemination needles, many injections of washed sperm resulted in very few outcross progeny. Therefore, we included data from only those that resulted in greater than 10 outcross progeny; these data support the hypothesis that male sperm cells themselves are competitively superior to hermaphrodite sperm.

In contrast to artificially-inseminated male spermatids, those taken from young adult, wild type hermaphrodites and injected into another herma-phrodite fertilized a relatively constant proportion of the offspring (figs 4A and 5). Unfortunately, herma-phrodite sperm were difficult to obtain, and therefore, fewer were loaded into the needles and injected into the *dpy-11* hermaphrodites, resulting in fewer outcross progeny ($\leq$27). Five injections of male sperm that produced <27 outcross progeny were used for comparison because we presumably injected few male sperm in these cases. Even though few male sperm were injected, they fertilized oocytes preferentially soon after injection and were mostly depleted within 1 day (fig. 4A). Thus, the fertilization pattern of injected hermaphrodite sperm is distinctly different from that of male sperm.

Discussion

In *C. elegans*, male sperm fertilize a greater share of the offspring soon after mating (or injection) than later. This pattern is the result of male sperm displacement[4]: newly introduced male sperm crawl to the spermatheca where, even if they are outnumbered by hermaphrodite sperm, they take precedence. Only after the male sperm are depleted do the hermaphrodite sperm begin to fertilize a greater portion of the offspring. These results, taken with those of earlier studies[4], refute the hypothesis that male sperm precedence is the result of numerical competition. When allowed to mate many times, some hermaphrodites subsequently produced only outcross progeny. In these cases, hermaphrodites received enough male sperm to effect complete and lasting displacement.

Results of our experiments show that male sperm cells are competitively superior to hermaphrodite sperm. Male sperm take precedence regardless of the length of time they have been activated, their mode of activation, or the presence of seminal fluid. Competitive superiority is limited, however, to male sperm; it is not a characteristic of any sperm introduced into the hermaphrodite, since hermaphrodite sperm artificially transferred to another hermaphrodite did not take precedence. Instead, the injected hermaphrodite sperm fertilized a constant share of offspring, presumably based on the number of sperm transferred. A similar lack of preference in fertilization was observed between the sperm of two males mated sequentially to self-sterile herma-phrodites[4]; each male sired a constant proportion of offspring. Thus, the competitive difference exists only between male and hermaphrodite sperm.

What might be the molecular mechanism underlying displacement? The interaction between male and hermaphrodite sperm provides some insight. It was observed earlier that male sperm displace hermaphrodite sperm from the walls of the spermatheca and take up residence where they will be the first to fertilize passing oocytes[4]. Thus, male sperm may adhere better to the spermathecal walls or may temporarily weaken herma-phrodite sperm. Because our present evidence indicates that the sperm cells themselves differ, we propose that the difference occurs on the cell surface where cell-cell and cell-spermatheca interactions are mediated. We are currently trying to identify genes involved in sperm precedence in *C. elegans* so that we may better understand its mechanism and its phylogenetic distribution among other, closely related nematodes.

The consistent competitive difference we have identified between sperm cell types in *C. elegans* appears to be unique. In insects, although various sperm morphs exist[12], the competitive ability of the fertilizing morphs themselves is not known to vary among individuals. Mating males must therefore compete for fertilizations either by inseminating greater numbers of sperm than the female's other mates[13,14] or by removing rival sperm from the reproductive tract of the female[15,16]. Alternatively, female insects may preclude competition

between ejaculates they receive either as a result of the shape of the spermatheca[17] or through selective sperm use[18,19]. In certain muroid rodents, the outcome of sperm competition among males varies consistently with laboratory strain[20]. However, competitive differences among these strains may simply be an effect of inbreeding which is known to correlate with elevated proportions of abnormal sperm[21]. Thus, *C. elegans* gender asymmetry in sperm cell competitiveness is novel but not surprising since outcrossing, which is maximized by sperm precedence, may reduce the costs of selfing.

There are, however, advantages to self-fertilization. Hermaphrodites may reproduce without having to find a mate and do not generate male offspring that by themselves cannot reproduce[22]. Thus, new habitats may be rapidly colonized by single individuals. Moreover, selfing organisms like *C. elegans* are spared many costs of asexual propagation. DNA damage that cannot be repaired in mitotic asexuals can be mended during meiotic recombination in *C. elegans*[23]. Also, asexual organisms suffer from Muller's ratchet, or the inevitable accumulation and fixation of deleterious mutations[24,25]. Such mutations can be segregated out during meiosis and syngamy in *C. elegans*, although some accumulation of mutations may occur in small, selfing populations[26]. One cost that cannot be avoided, however, is the increase in homozygosity that results from self-fertilization. After many generations of self-fertilization, individuals become predominantly homozygous with little genetic variation among individuals[5]. When new habitats are colonized by few individuals, selfing would lead to genetically invariant populations. Such a lack of variation is thought to predispose populations to extinction[21,27,28].

Therefore, even though males occur infrequently (1 in 500 or less), mating likely plays an important role in maintaining genetic variation in natural populations of *C. elegans*. Outcrossing may, in fact, become an important means of reproduction when temperatures approach the upper thermal limits for *C. elegans*. Under such thermal stress, male production rises ten-fold[29]. Increasing temperature may portend environmental change such as the onset of a dry season through which the offspring would pass in the resistant, dauer stage and subsequently find themselves in a new environment. Alleles that function normally in the parents' environment may be deleterious in the new environment. Such a deleterious allele was found in a natural strain of *C. elegans* isolated in France: fertility is normal at lower temperatures but severely reduced at higher temperatures that are well within the natural thermal limits for other *C. elegans*[30]. Mating produces heterozygotes and thereby masks the effects of potentially deleterious alleles. However, mating would not be as effective if sperm displacement did not occur. Even when relatively few sperm are transferred during mating, these sperm take precedence and fertilize oocytes immediately.

Sexual dimorphism in *C. elegans* sperm cell competitiveness may therefore be an adaptive trait that maximizes outcrossing after mating in a primarily hermaphroditic species. Hermaphroditism is probably derived in *Caenorhabditis*[31] since male/female nematode species outnumber hermaphroditic ones[32]. Thus, the observed competitive difference between sperm cells may have evolved either during or soon after the evolution of the hermaphroditic life cycle as a means for masking deleterious alleles and/or increasing genetic diversity in offspring.

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