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Evolution of larger sperm in response to experimentally increased sperm competition in *Caenorhabditis elegans*

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Sperm morphology evolves rapidly, resulting in an exceptional diversity of sperm size and shape across animal phyla. This swift evolution has been thought to prevent fertilizations between closely related species. Alternatively, recent correlative analyses suggest that competition among sperm from more than one male may cause sperm diversity, but these hypotheses have not been tested. Here, we test experimentally the effect of sperm competition on sperm-size evolution using the nematode *Caenorhabditis elegans*. This worm has a three day generation time, which allowed the study to cover many generations. Sperm volume increased nearly 20% over 60 generations in lines genetically induced to have high levels of sperm competition compared with those of control lines. These results show that sperm competition can and does cause morphological evolution of sperm and, therefore, can explain much of the diversity in sperm morphology.

Keywords: sperm competition; sperm evolution; sperm size; nematodes; *Caenorhabditis elegans*

1. INTRODUCTION

Sperm are exceptionally variable in morphology. Sperm types range from the familiar tadpole-like cells in mammals, to multiflagellate cells in termites, to disc-like cells in proturans, to amoeboid cells in nematodes (Sivinski 1980; Simmons & Siva-Jothy 1998). Such extensive morphological variation is not seen among ova (Joly *et al.* 1991). Even within closely related species, sperm may vary widely in shape and size. Among *Drosophila* for example, sperm length varies by more than two orders of magnitude (Pitnick *et al.* 1995; Snook 1997), implying extremely rapid evolution. Indeed, sperm are so rich in variation that they are commonly used to distinguish evolutionary relationships among taxa (Jamieson 1991; Jamieson *et al.* 1995; Guidi & Rebecchi 1996), however the cause of this variation is unclear.

Two different hypothetical forces may have caused the evolution of sperm variation. The first is species isolation. During and after speciation, both the female reproductive tract and sperm morphology diverge, and sperm from one species will not fit properly into the female reproductive tract of the other species, preventing fertilization (Dybas & Dybas 1981; Birkhead 2000). Indeed, evidence is now accumulating that sperm coevolve with female reproductive tract morphology (Dybas & Dybas 1981; Pitnick *et al.* 1999; Presgraves *et al.* 1999), but this correlation is thought to be due instead to the second hypothetical force, post-copulatory sexual selection (Parker 1970). Proper fit within the female reproductive tract could give an advantage to sperm when in competition with sperm from conspecific males. In some cases, multitudes of tiny, inexpensive sperm will probably be most competitive (Parker 1982; Tuttle *et al.* 1996), but in other cases, the more competitive sperm morphologies may be large and costly to produce (Radwan 1996; LaMunyon & Ward 1998). Taxa experience different risks of sperm competition due to variation in the tendency of females to mate with multiple partners, and where competitive morphologies are expensive, the degree of morphological evolution may depend upon the risk of sperm competition. Comparative studies have shown that sperm size increases with greater risk of sperm competition in some taxa (Gomendio & Roldan 1991; Gage 1994; Briskie *et al.* 1997; but see Harcourt (1991) and Hosken (1997)). However, owing to the difficulties of performing long-term evolutionary experiments, the effects of sperm competition on sperm morphological evolution have not, to our knowledge, been tested empirically.

Here, we report an experimental investigation of the causal link between sperm competition and sperm-size evolution in *Caenorhabditis elegans*. Sperm size is the single most divergent character in rhabditid nematodes (which includes *C. elegans*), varying from 6 to 1089 μm^3 (LaMunyon & Ward 1999). Nematode sperm are amoeboid, and in *C. elegans*, larger sperm crawl faster and exclude smaller sperm from the spermatheca, the sole site of fertilization (LaMunyon & Ward 1998). However, larger sperm are not without cost: they take longer to produce (LaMunyon & Ward 1998). Across the family Rhabditidae, larger sperm occur in species with greater risk of sperm competition (LaMunyon & Ward 1999), suggesting that pressure from sperm competition has resulted in the evolution of larger sperm.

To test whether larger sperm evolve in response to sperm competition, we manipulated the risk of sperm competition by controlling the mode of reproduction in experimental *C. elegans* lines. Three lines were forced to reproduce as male/female populations using a mutation in the *spe-8* gene (allele *hc53*, which renders hermaphrodites self-sterile, but has little effect on male fertility; Shakes & Ward 1989). In these sperm competition lines, (SC+ lines), males were abundant, hermaphrodites were multi-

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ply mated, and the risk of sperm competition was great. Three other lines were maintained by strict self-fertilization (SC− lines), where no sperm competition occurred. Sperm-size evolution was followed in all these lines for 60 generations.

2. METHODS

Worms were provided by the Caenorhabditis Genetics Center and grown at 20 °C in Petri plates on agar seeded with a lawn of the *Escherichia coli* strain OP50. The agar was made with a modified formula of Nematode Growth Medium (NGM) (Brenner 1974): to each litre of agar we added 8.0 g of tryptone and 5.0 g of yeast extract (NGMYT agar). This formula was better for OP50 growth. The plates were coated with NGMYT agarose to prevent the worms from burrowing.

To construct the evolving lines, four wild isolate strains were used (CB4855, DR1345, DR1350 and AB1) in combination with an N2 strain homozygous for the *spe-8*(*hc53*) mutation. Hermaphrodites from the strain *spe-8*(*hc53*) are self-sterile because their spermatids fail to mature into spermatozoa. Male *spe-8*(*hc53*) are normal. The *spe-8*(*hc53*) mutation was crossed into each of the other four wild isolate strains by the following protocol: male *spe-8*(*hc53*) were mated to hermaphrodites from each of the other four strains. The heterozygous F_1 progeny were paired for sib matings, and the F_2 hermaphrodite progeny were isolated as juveniles to ensure their virginity and checked for sterility (60 worms per cross; 240 worms in total). Sterile F_2 hermaphrodites (homozygotes for *spe-8*(*hc53*)) were then each paired with a single F_2 sib male (genotype unknown) and allowed to mate. Eight F_3 hermaphrodite progeny from each of these crosses were then isolated and checked for sterility. Twelve lines that produced sterile F_3 hermaphrodites (three from each original P_0 cross) were retained as homozygous *spe-8*(*hc53*)/wild isolate strains for the SC+ lines. In addition, 12 lines that produced fertile F_3 hermaphrodites (heterozygous for *spe-8*(*hc53*)) were retained for the SC− lines.

The SC+ lines were constructed by combining five hermaphrodites and 10 males from each of the 12 homozygous *spe-8*(*hc3*)/wild isolate lines into one population. Three such lines were initiated. The three SC− lines were founded by combining five hermaphrodites from each of the 12 heterozygous *spe-8*(*hc53*)/wild isolate lines. To found each new generation, 60 hermaphrodite and, in the SC+ lines, 100 male progeny, were transferred as L4 larvae to fresh plates. Because it was critical that each new generation was established with progeny fertilized by sperm that were in competition, the interval between transfers was 4 days. The transferred progeny were produced one full day after their parents were paired and thus were likely to be fertilized by sperm that were in competition.

Twenty males from each of the six lines at generations P_0, F_{15}, F_{30}, F_{45} and F_{60} were taken for sperm-size measurements. These males were isolated as last stage larvae (L4), left for 15–20 h to molt to adults, and dissected under Sperm Medium (LaMunyon & Ward 1998). Males store spermatids, spherical cells that extend a pseudopod to become spermatozoa that retain the same volume they had as spermatids (Roberts *et al.* 1986). Images of the spermatids were captured under Nomarski optics, and the cross-sectional area of approximately 20 spermatids from each male were measured using NIH Image (LaMunyon & Ward 1998). Spermatid volume was calculated as that of a sphere, which the spermatids approximate. To reduce subjectivity, sperm were measured without knowledge of their strain of origin: each digital image of sperm at each generation was assigned a random number. At each generation approximately 250 images were captured from all lines. Thus, the identity of the sperm (SC+ or SC−) was unknown during measurement.

While males were readily available from the SC+ lines, they were nearly absent in the selfing lines. Self-fertilization gives rise to males at a frequency of 1 in 500 progeny through infrequent non-disjunction of the X chromosome: hermaphrodites, XX; males, XØ (Hodgkin 1983, 1988). The SC− plates were scanned for infrequent males until at least six were found. These males were placed with 10 arbitrarily selected hermaphrodites from the same plate, allowed to mate, and resulting male progeny paired with additional hermaphrodites from their line. The male progeny from these second crosses were used in the sperm-size measurements, and represented an attempt to sample broadly from the population of each SC− line. In total, 6384 sperm were measured.

Male body size was measured by placing males in a depression slide in an isotonic buffer containing sodium azide as an anaesthetic (LaMunyon & Ward 1994). After a 15 min exposure to the anaesthetic, the worms had ceased movement, and images were captured under light microscopy. The body silhouette area was measured with NIH Image.

3. RESULTS

The genetic wild-type strain of *C. elegans* (var. Bristol N2) bearing the *spe-8*(*hc53*) mutation has been inbred in the laboratory for many generations by self-fertilization, and therefore it is likely to have little or no genetic variation. Selection will not result in evolution without genetic variation in the selected trait, so we constructed the evolving lines by crossing the *spe-8*(*hc53*) mutation into four 'wild-isolate' strains, chosen on the basis of variation in their sperm sizes (figure 1). Wild isolates have been collected from diverse geographical localities but have not been manipulated genetically. The fact that sperm size varied among these strains, even when raised under identical conditions, indicates that the variability in sperm size is genetic.

Over the course of 60 generations, larger sperm evolved in the SC+ lines: male sperm volume increased by nearly 20% (figure 2; ANOVA comparing generation means: $F_{4,244} = 9.307$, $p < 0.00\ 001$). The SC− lines did not show such evolution of male sperm size (figure 2; ANOVA: $F_{4,244} = 1.463$, $p > 0.05$). These results support the hypothesis that sperm competition causes evolution of larger sperm. However, at the end of the 60 generations, males in the SC+ lines were significantly larger than males in the SC− lines (mean body silhouette area ± s.e.m.: SC+ lines, 31 707 μm² ± 339; SC− lines, 27 410 μm² ± 351; $F_{1,112} = 77.7$, $p < 0.0001$). If larger males had an advantage in the competition for mates, and body size and sperm size were correlated, then larger sperm could have evolved not because they provided an advantage, but because of their allometric relationship with body size. To distinguish between these two hypotheses, we checked the relationship between body size and sperm size in the SC+ lines. We found no correlation between male body size and sperm size (raw data: $r = 0.160$, $p > 0.05$; log-transformed data: $r = 0.179$, $p > 0.05$). Thus, sperm size did not evolve as a consequence of selection on male body

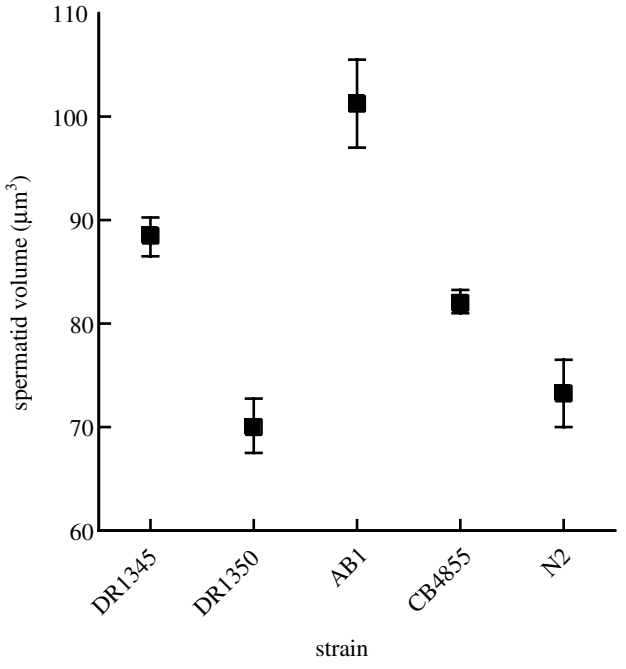

Figure 1. Male sperm size in five strains of *Caenorhabditis elegans*. The volume was calculated from the cross-sectional area of spermatids, the immature spherical precursors of spermatozoa. Sperm from eight males from each strain were measured except for CB4855, where 14 males were used. For each male, 18–30 randomly chosen sperm were measured. Error bars, ±1 s.e.m.

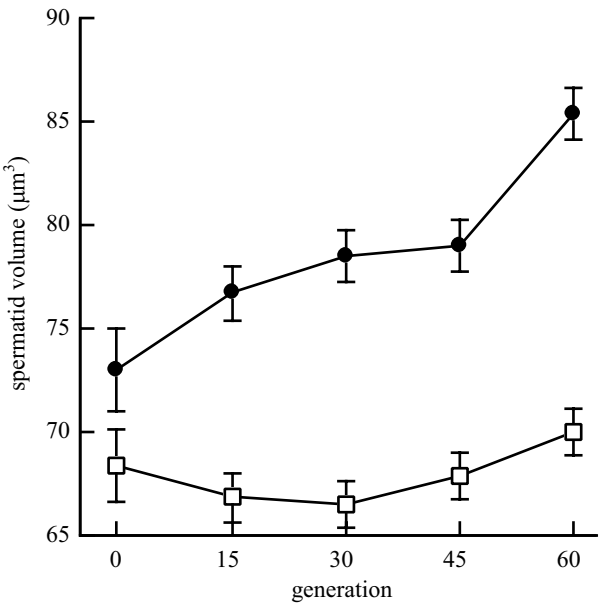

Figure 2. Evolution of male sperm size over 60 generations. Closed symbols represent the average of the three SC+ lines where sperm competition was intense. Open symbols represent the average for the three SC− lines where there was no sperm competition. For each line at each time-point, 20 randomly chosen sperm from each of 20 males were measured. Error bars, ±1 s.e.m.

size, but rather as a direct response to selection by sperm competition.

4. DISCUSSION

These results have important implications for understanding the vast morphological variability of sperm. If fertilization were the only function sperm must perform, we would expect sperm to be no more variable than ova. However, in addition to fertilization, sperm must also compete with non-sib sperm, a consequence of female multiple mating. While sperm competition occurs in nearly every animal species (Smith 1984; Birkhead & Møller 1992, 1998), the risk varies depending upon the tendency of females to take multiple mates. Another variable is the female reproductive tract, where the differences among species select for different sperm morphologies that maximize competitiveness. The extensive variation in sperm morphology is probably a reflection of both the risk of sperm competition and the environment in which it takes place. In many cases, this will result in the evolution of many small sperm (Parker 1982), which may be the case in Australian fairy-wrens, where sperm are minute and sperm counts number in the billions (Tuttle *et al.* 1996). In other species, different morphologies might arise, such as sperm with a corkscrew shape that apparently facilitates competition for access to the spermatheca in the featherwing beetles (Dybas & Dybas 1981). In nematodes, the competitive environment gives larger sperm the advantage, and in species with intense sperm competition, larger sperm are present (LaMunyon & Ward 1998). Where larger sperm are more competitive, intense sperm competition may drive evolution of sperm so large that males are sperm limited. Such may be the case in the nematode *Rhabditis brassicae*, where the sperm are 20 times larger in volume than those of *C. elegans* males (LaMunyon & Ward 1999), but are produced at a rate of only nine per hour, compared with 60 per hour in *C. elegans* (LaMunyon & Ward 1998). In *Drosophila*, males that produce giant sperm are also sperm limited (Pitnick 1996).

It is interesting that in addition to sperm size, male body size also increased in the SC+ lines. While our results show that larger males do not produce larger sperm, they may produce sperm at a greater rate, which would offset the sperm limitation imposed by the evolution of larger sperm in the SC+ lines. It is well known that larger male insects produce larger ejaculates (Wedell 1997), so it is not unlikely that the same holds true for other invertebrate taxa, including nematodes. Whatever the ultimate reason for the increase in body size, the fact that intense mating and sperm competition can drive the evolution of a larger soma is of considerable importance. Body size evolution is apparently constrained in soil nematodes, due most likely to the need to move efficiently among soil particles. Worms in the family Rhabditidae (to which *C. elegans* belongs) are very similar in body size, even though DNA sequence variation in the family is fivefold greater than that found among the most divergent tetrapod classes (Fitch & Thomas 1997). Therefore, sexual selection in nematodes via mating and/or sperm competition may drive the evolution of male bodies that are not well adapted for movement through the soil.

The effects of sexual selection on traits that influence the likelihood of mating are well known. However, the effects of sexual selection acting during and after mating are only now being recognized as pervasive. Such effects

include the evolution of toxic ejaculates (Rice 1996; Prout & Clark 2000), genitalic complexity (Arnqvist 1998), testis size (Hosken *et al.* 2001) and sperm morphology as we have demonstrated here and as has been suggested for other species (Gomendio & Roldan 1991; Gage 1994; Briskie *et al.* 1997; Morrow & Gage 2000).

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