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Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*

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Sperm competition is generally thought to drive the evolution of sperm miniaturization. Males gain advantage by transferring more sperm, which they produce by dividing limited resources into ever smaller cells. Here, we describe the opposite effect of size on the competitiveness of amoeboid sperm in the hermaphroditic nematode *Caenorhabditis elegans*. Larger sperm crawled faster and displaced smaller sperm, taking precedence at fertilization. Larger sperm took longer to produce, however, and so were more costly than smaller sperm. Our results provide evidence of a mechanism to support recent theoretical and comparative studies that suggest sperm competition can favour not small, but large sperm.

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

1. INTRODUCTION

It is widely held that sperm miniaturization is advantageous. Initially, sperm and eggs were favoured by selection for both smaller, more numerous proto-sperm and larger, high-survival proto-eggs over gametes of intermediate size (Parker *et al*. 1972). If sperm from several individuals compete to fertilize eggs, increased sperm number is especially beneficial. Indeed, males of a number of species, including humans, pass more sperm when the risk of sperm competition is greatest (Gage 1991; Gage & Baker 1991; Baker & Bellis 1993; Gage & Barnard 1996). Increased sperm numbers leads to selection for further sperm miniaturization as limited resources are divided into ever smaller units (Parker 1982, 1984). Thus, the numerical superiority afforded by making small sperm may give males a competitive edge. However, theoretical and comparative studies have also suggested that larger sperm may evolve as a response to sperm competition (Gomendio & Roldan 1991; Parker 1993; Gage 1994; Briskie *et al*. 1997).

We investigated the possibility that sperm size determines the outcome of competition among the amoeboid sperm of the hermaphroditic nematode *C. elegans*. In this species, hermaphrodites resemble females but undergo a brief period of spermatogenesis before switching irreversibly to oogenesis and undergoing self-fertilization. Males are scarce but mate readily with hermaphrodites to produce cross progeny (Honda 1925). Nematode sperm compete to fertilize eggs by vying to gain access to the spermatheca and then to maintain residence in this organ where the eggs are fertilized. Male sperm always outcompete self-sperm from hermaphrodites (Ward & Carrel 1979; LaMunyon & Ward 1995), but compete equally with sperm from other males (Ward & Carrel 1979; Barker 1994). The mechanism of male sperm precedence over hermaphrodite self sperm does not involve sperm age or seminal fluid and has remained unclear (LaMunyon & Ward 1995). However, male–male sperm competition is thought to be a 'fair raffle' (Parker *et al*. 1990) in which numerical representation determines paternity (Ward & Carrel 1979; Barker 1994). In this study, we found that sperm size did indeed determine sperm competitiveness. Larger sperm crawled faster, displaced smaller sperm from the spermathecae, and took fertilization precedence, but not without a cost: they took longer to produce.

2. METHODS

(a) *Sperm size*

Our experiments were performed on worms from strains provided by the *Caenorhabditis* Genetics Center (CGC) and maintained in the laboratory in Petri plates on agar seeded with the *E. coli* strain OP50 at 20–23.5 °C (Brenner 1974). Measurements of sperm size were taken from spermatids, the haploid gametes that sprout a pseudopod and become mature spermatozoa. As a result of their irregular shape (figure 1*a*), spermatozoa are difficult to measure but retain the same volume they had as spermatids (Roberts *et al*. 1986), which are spherical and easy to measure (figure 1*b*,*c*). Worms were dissected in sperm buffer (Nelson & Ward 1980) and observed under Nomarski optics. The cross-sectional area of the spermatids from one-day-old virgin males and from freshly moulted adult hermaphrodites was measured from video-captured images using the analysis software NIH Image. We also measured the size of sperm within the spermathecae and uteri of reproductive tracts dissected out of mated hermaphrodites. The maximal diameter of the sperm cell bodies was measured in a direction perpendicular to the direction of the pseudopod (figure 1*a*) by a person who did not know the location of the sperm (uterine or spermathecal). These measures were unlikely to be affected by distortion due to the passage of eggs through

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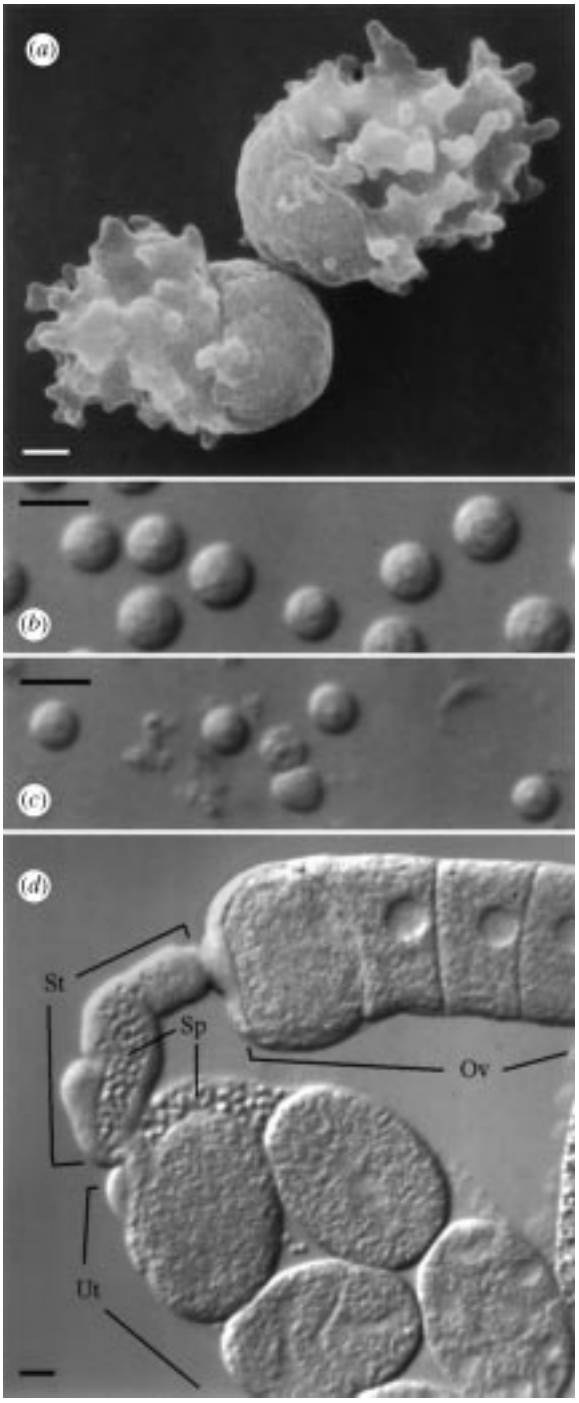

Figure 1. (*a*) Scanning electron micrograph of spermatozoa. Bar, 1 μm. (*b*) Male spermatids. (*c*) Hermaphrodite spermatids. Bars in (*b*, *c*), 5 μm. (*d*) Composite micrograph of a mated hermaphrodite reproductive tract. Eggs pass from the oviduct (Ov) through the spermatheca (St) where fertilization occurs and then into the uterus (Ut) before being laid through the vulva. Note the presence of sperm (Sp) in both the spermatheca and the uterus. Bar, 10 μm.

the two organs, as the sperm seem to retain their normal shape no matter where they are located.

(b) *Sperm motility*

Male sperm, which males store as spermatids, were induced to form pseudopodia using the weak base triethanolamine (Ward *et al*. 1983); the resulting active spermatozoa crawl and are capable of fertilization (LaMunyon & Ward 1994). Hermaphrodites' self-spermatozoa are lodged within the spermathecae where very few are available for analysis. Therefore, we used *spe-8*(*hc53*) hermaphrodites in which the self sperm remain available as spermatids that can be activated by triethanolamine treatment. To control for the effects of the *spe-8* mutation, we analysed sperm from *spe-8*(*hc53*) males in addition to sperm from wild-type males. In all, 42 sperm from six wild-type males, 32 sperm from four *spe-8* males, and 13 sperm from four *spe-8* hermaphrodites were analysed. Motility rates were estimated from videotape recordings of sperm crawling on poly-L-lysine-subbed glass slides under Nomarski optics.

(c) *Male–male sperm competition*

Hermaphrodites were mated sequentially to males from two strains. The hermaphrodites were homozygous for two mutations derived from the genetical wild-type strain N2: (i) *spe-12*(*hc76*), which blocks spermatid maturation and thereby removes the hermaphrodite self-sperm from the competition; and (ii) *dpy-20*(*e1282ts*), which causes a dumpy (short, fat) morphological phenotype used for paternity assignment (Hodgkin *et al*. 1988). The hermaphrodites were always mated to males from the N2 derivative strain *dpy-20*(*e1282ts*) *him-5*(*e1490*). (The *him-5* mutation increases male production in unmated hermaphrodites and was used here to facilitate collection of males for experiments.) The rival males came from the strains N2 or ABl (collected in Australia) and were mated to the hermaphrodites either as first or second mates. Hermaphrodites, isolated in the last juvenile stage, were kept as virgins for 40 h to allow the defective self-sperm to be removed by passing unfertilized oocytes (L'Hernault *et al*. 1988), and then paired with males in 35-mm plates for 3 h in a ratio of 4–6 hermaphrodites to 12–18 males. After isolation for 1 h, those that laid fertile eggs were paired with males from another strain in the same manner as the first mating. Thus the hermaphrodites were mated by one or more males from each strain, placing the sperm from each strain in competition. In total, 115 hermaphrodites were mated by males from the two strains. The hermaphrodites were subsequently placed individually on plates where they continued to lay eggs, and they were transferred to fresh plates at regular intervals.

Paternity was assigned on the basis of the dumpy phenotype. When adult, the hermaphrodite progeny were scored as either dumpy (sired by the dumpy males), or wild-type (sired by the N2 or ABl males). The phenotype of the male progeny was not as obvious, so they were not scored, but a total progeny count was recorded. Eighteen hermaphrodites died prematurely (within 42 h of mating) and were omitted. The remaining 97 hermaphrodites produced an average of 347 progeny (s.d. = 123). Of these, 19 showed complete sperm precedence by one of the male strains. (Eleven had complete second-male precedence indicating that few sperm were transferred during the first mating; eight showed first-male precedence, perhaps because they did not received a second mating.) These 19 were also omitted from the analysis.

(d) *Sperm production*

Plates containing males in the last juvenile stage were observed at 15-min intervals, and any males that had completed the adult moult were fixed either immediately or after an interval of 2 h and stained with the DNA label DAPI (Sulston & Hodgkin 1988). Specimens were flattened under a coverslip until all the sperm nuclei were within one plane of focus under

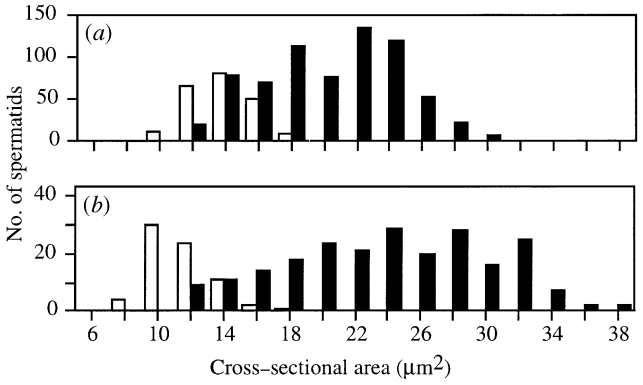

Figure 2. Distribution of spermatid sizes for strains AB1 (*a*) and N2 (*b*). Male spermatids (filled bars) differed significantly between strains (nested ANOVA, 430 sperm from 16 worms: $F_{1,414}=90.6$, $p<0.001$), as did the hermaphrodite spermatids (open bars; nested ANOVA, 292 sperm from 28 worms: $F_{1,264}=16.5$, $p<0.001$).

epifluorescence, and sperm nuclei were counted on videotaped images of the specimens.

3. RESULTS

(a) *Sperm size*

Spermatids from males were much larger on average than those from hermaphrodites (figures 1*b*,*c* and 2). Male spermatids also varied greatly in size, ranging from that of hermaphrodite spermatids to more than twice their size in cross-section (figure 2). Although we used cross-sectional area for ease of measurement, the actual volumetric differences would be greater. Sperm size also varied between the two *C. elegans* strains examined: the Australian strain ABl had significantly larger male spermatids than did N2, which was isolated in England (figure 2).

To determine whether the larger sperm take up residence in the spermathecae preferentially, we compared the cell-body diameter of 119 spermathecal sperm (mean±s.e., 4.26±0.05 μm) and 120 uterine sperm (mean±s.e., 3.89±0.05 μm), from the reproductive tracts of nine mated hermaphrodites similar to that shown in figure 1*d*. Although sperm size varied significantly among worms ($F_{9,219}=3.03$, $p=0.002$), spermathecal sperm were significantly larger than uterine sperm ($F_{1,219}=19.58$, $p<0.001$). Thus, larger sperm do take over the prime locations for fertilization.

(b) *Sperm motility*

Larger sperm crawled faster than did smaller sperm (figure 3). In fact, the largest male sperm crawled approximately ten times faster than did the smallest hermaphrodite sperm, which, at 3 μm min^{-1}, were nearly immotile (figure 3). Sperm crawl when projections on the pseudopod attach to the substrate and treadmill from the tip of the pseudopod toward the cell body, pulling the cell in the direction of the pseudopod (Ward *et al*. 1983) (figure 1*a*). *In vitro*, some sperm attach to the substrate by their pseudopodia and crawl; for other sperm, the pseudopodia never attach even though projections treadmill from the tip of the pseudopod to the base. Our estimates of motility included both direct measures of crawling sperm and, when the sperm's pseudopod did not attach, measures of pseudopodial treadmilling (averaged from the movement of two pseudopodial projections for each sperm), the rate of which is known to be equivalent to crawling rate (Ward *et al*. 1983; Roberts & King 1991). Although the sperm from individual worms varied greatly in size and motility, there were significant differences among worms ($F_{13,73}=10.62$, $p<0.001$). An analysis of covariance of residual variation in crawling rate that was not explained by differences among worms showed that neither the genotype of the worm (N2 or *spe-12*: $F_{1,82}=0.11$, $p=0.74$) nor the type of measure (crawling or treadmilling: $F_{1,82}=0.029$, $p=0.86$) had an effect on the residual rate of motility. Only the covariate sperm diameter had a significant effect ($F_{1,82}=5.16$, $p=0.02$). In addition to crawling faster, larger sperm were significantly more likely to have their pseudopodia attach to the substrate *in vitro* ($F_{1,87}=8.08$, $p=0.006$).

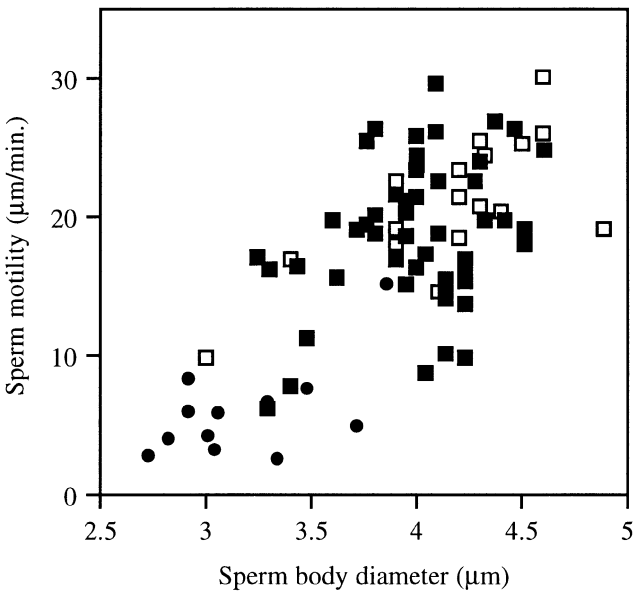

Figure 3. Sperm motility as a function of sperm size. Open boxes, male sperm that crawled; filled boxes, male sperm with pseudopodial treadmilling; circles, hermaphrodite sperm with pseudopodial treadmilling. The correlation between sperm size and motility was significant ($r=0.70$, $p<0.001$, $n=87$).

(c) *Sperm competition*

To further investigate the effect of sperm size on fertilization precedence, we examined the paternity of offspring from hermaphrodites mated to males derived from strains ABl and N2. Approximately 16% of the ABl sperm were larger than those from N2 males (figure 2). If, contrary to the hypothesis, sperm size is not important, then offspring paternity will depend upon the relative numbers of sperm present and remain constant over the life of the hermaphrodite, assuming there are no strain effects on sperm mortality. If, on the other hand, the hypothesis is correct, the largest ABl sperm should fertilize eggs immediately, resulting in greater ABl paternity in the early progeny. Thus, the critical feature is the comparison of paternity in the early versus the later progeny. Indeed, when ABl males mated second, their sperm took immediate precedence, fertilizing significantly more of the early progeny than the later progeny (figure 4). In contrast, ABl males had no such advantage when the

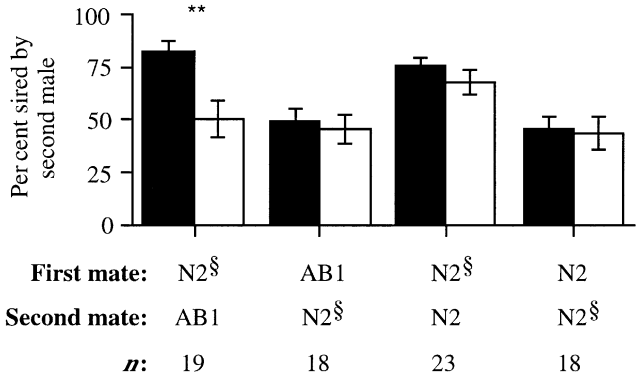

Figure 4. Competition between male sperm from the strains AB1 and N2. To assess sperm competitiveness, the percentage of the offspring sired by the second male is compared between the early progeny (laid during the first 18 h after the second mating; filled bars) and the late progeny (from the final 24 h of oviposition or the final 48 h if fewer than ten hermaphrodite offspring were produced during the final 24 h; open bars). The N2 strain carrying the morphological marker *dpy-20*(*e1282ts*) is denoted by the symbol §. *n* is the sample size, error bars represent 1 s.e.m. Early versus late progeny were statistically treated by paired *t*-test after arcsine transformation of the data. Statistical probabilities were corrected for multiple comparisons using the Bonferroni technique (Rice 1989); **, $p=0.0002$; all other pairs not significant.

hermaphrodites mated with them first (figure 4). This result is consistent with larger sperm having an advantage, as the largest, most competitive sperm of the AB1 males would be used first, during the interval between matings.

To assess the effect of the genetic marker, *dpy-20*(*e1282ts*), on sperm competitiveness, control matings were carried out, pitting male sperm from the marked N2 strain against male sperm from the wild-type N2 strain. Paternity of the early progeny was no different from that of the later progeny (figure 4), indicating that sperm from the two strains were equally competitive. However, when the wild-type males mated second in the control matings, they attained greater paternity than did the marked males as second mates (figure 4). This shows that, even though the sperm from marked males were fully competitive, they were at a numerical disadvantage, either because marked males pass fewer sperm at mating, or because they mate at a reduced rate.

(d) *Sperm production*

Larger sperm are more competitive, but is there a cost to making large sperm? One potential cost is the investment of resources per sperm, which we assessed by measuring the rate of male sperm production in strains N2 and AB1. At the adult moult, AB1 males contained 179 sperm (s.e.m.=7, $n=25$); 2 h after the moult, they had 270 (s.e.m.=8, $n=22$), which gives a rate of production of 45 sperm per hour. N2 males had 163 sperm (s.e.m.=6, $n=29$) at the adult moult, and 284 (s.e.m.=6, $n=29$) 2 h after the moult, giving a rate of 60 sperm per hour. Thus, the cost to AB1 males of making larger sperm is a significant reduction in the rate of sperm manufacture compared to that of N2 males (ANOVA, interaction between strain and time: $F_{1,101}=4.457$, $p=0.037$).

4. DISCUSSION

Our results indicate that sperm competitiveness is a function of sperm size in *C. elegans*. Male sperm are known to take precedence over hermaphrodite sperm (Ward & Carrel 1979; LaMunyon & Ward 1995), and here we show that male sperm are larger than hermaphrodite sperm. To take precedence after being ejaculated into the uterus, male sperm must crawl up either arm of the bilobed hermaphrodite reproductive tract to the spermathecae, the sites of fertilization (Ward & Carrel 1979) (figure 1*d*). There they encounter and must displace the hermaphrodite self-sperm, which accumulate in the spermathecae before the hermaphrodite can mate. As eggs pass through the spermathecae, some sperm are swept down into the uterus and must crawl back into the spermathecae to participate in fertilization. Male sperm could gain an advantage by crawling faster or by clinging more tightly to the substrate, both of which they did in our experiments *in vitro*. Our measures of sperm in the reproductive tracts of mated hermaphrodites showed that the larger sperm do take up residence in the spermathecae; the smaller hermaphrodite sperm are displaced into the uterus where they risk being lost through the vulva as the eggs are laid. Indeed, nearly all the hermaphrodite sperm can be lost when hermaphrodites receive an abundance of male sperm as observed by Ward & Carrel (1979).

A more direct test of the effect of sperm size on competitiveness was the competition between male sperm that differed in their range of sizes. Sperm from AB1 males had an early advantage over N2 sperm from a previous mating, but not from a subsequent mating. A proportion of sperm from AB1 males were larger than those from N2 males. It is likely that these largest AB1 sperm took immediate precedence over the smaller pre-existing rival sperm, but they would have already fertilized eggs by the time the subsequent rival sperm arrived. Although we cannot rule out other strain effects on our results, sperm age is unlikely to have been important. The sperm differed in age by only several hours but competed over the course of several days. Furthermore, sperm age does not play a role in male–hermaphrodite sperm competition (LaMunyon & Ward 1995). Thus, these results support the hypothesis that sperm size is a component of ejaculate competitiveness. Male–male sperm competition in *C. elegans* is therefore more complex than was originally thought. Earlier work suggested that male sperm are competitively equal, and their numbers determine paternity (Ward & Carrel 1979; Barker 1994). In our experiments, larger male sperm outcompeted smaller male sperm, but they seemed to compete equally with sperm of similar size. Thus, variation in sperm size helps explain the patterns of paternity observed in this species.

Although larger sperm are more competitive, they also cost more to produce, measured here as a reduced rate of manufacture. The relative effects of these two selective pressures have probably had an important impact on the evolution of sperm size in *C. elegans*. Hermaphrodites make small, non-competitive sperm, which indicates that the benefits of making large sperm have been outweighed by the costs. In fact, there may be no benefit to increased

competitiveness for hermaphrodite sperm, as it may be to the hermaphrodite's advantage to 'allow' male sperm to take precedence. Self-fertilization is the ultimate form of inbreeding, and outcrossing may provide a number of benefits to these self-fertile hermaphrodites (e.g. complementation of deleterious alleles, increased genetic variability, etc.) that are maximized by allowing male sperm to take precedence. Moreover, sperm production rate is very important to hermaphrodites, which are under intense pressure to proceed quickly through spermatogenesis and thereby reduce the time to egg laying, an important component of the ability to colonize new habitats rapidly (Hodgkin & Barnes 1991). Producing small sperm increases the rate of spermatogenesis. Thus, the prevailing selective pressures are for small hermaphrodite sperm. However, the smallest hermaphrodite sperm were barely motile (figure 3); if they were any smaller, they probably could not crawl. Therefore, hermaphrodite sperm may have reached a lower size limit, given the constraints of sperm motility.

The benefit of increased competitiveness has apparently been important in the evolution of male sperm size. At mating, male sperm always encounter hermaphrodite sperm (unless the hermaphrodite sperm have become depleted) but are much less likely to encounter other male sperm because males are scarce, typically comprising less than 1% of populations (Honda 1925). Thus, male–hermaphrodite sperm competition is the most important form of sperm competition in *C. elegans* and has probably selected for the evolution of male sperm that are larger than hermaphrodite sperm. However, as male–male sperm competition is so rare, there would seem to be little benefit in producing even larger sperm, given the trade-off with sperm production rate. Although male–male sperm competition apparently has not been important in the evolution of sperm size in *C. elegans*, it has been important in gonochoristic (male/female) nematode species, where males comprise 50% of populations and where male sperm are much larger than male *C. elegans* sperm, even though male body size is almost identical among species (C. W. LaMunyon and S. Ward, unpublished data).

Our results identify both a mechanism and a cost of a large-sperm advantage, supporting recent theoretical and comparative studies indicating that production of larger sperm can provide a benefit in the face of sperm competition (Gomendio & Roldan 1991; Parker 1993; Gage 1994; Briskie *et al*. 1997). Others have found a positive intraspecific correlation between sperm size and either fertilization priority (amoeboid arachnid sperm: Radwan 1996), or preferential sperm storage (flagellated insect sperm: Otronen *et al*. 1997), but the mechanism and cost of the size advantages remain unclear. Taken together, these studies indicate that sperm competition frequently results in the evolution of large sperm, especially where the sperm themselves are not passive 'lottery tickets', but instead compete actively for fertilizations.

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