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Abstract

Contrary to early predictions of sperm competition theory, postcopulatory sexual selection favoring increased investment per sperm (e.g., sperm size, sperm quality) has been demonstrated in numerous organisms. Recent findings reveal that sperm production strategies are highly variable, with males of some species producing relatively few, giant sperm. We empirically demonstrate for *Drosophila melanogaster* that both sperm quality and sperm quantity independently contribute to competitive male fertilization success. The interaction between sperm quality and quantity suggests an internal positive reinforcement on selection for sperm quality, with selection predicted to intensify as investment per sperm increases and the number of sperm competing declines. The mechanism underlying the sperm quality advantage is elucidated through examination of the relationship between female sperm-storage organ morphology and the differential organization of different length sperm within the organ. Our results exemplify that primary sex cells can bear secondary sexual straits.

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Acknowledgements

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Introduction

Studies indicate postcopulatory sexual selection exists in certain female types creating a bias in favor of long sperm (Pitnick 1999, Briskie 1997, Gage 1994). This is directly opposed to the accepted dogma that a winning strategy for fertilization success is for males to produce high numbers of low-investment (small size) gametes. Since previous studies have clearly shown not only an increase in fertilization success with increasing sperm number, as would be expected, but also an increase with sperm size in some systems, we set out to compete these antagonistic traits against each other. It was recently suggested that the long sperm tails of *Drosophila* are the cellular, postcopulatory equivalent of peacock tails (Miller and Pitnick 2002). A compelling body of evidence supports this contention (Keller and Reeve 1995; Snook 2005). First, sperm cells are the most diverse cell type known, exhibiting rapid and dramatic evolutionary divergence in form (Baccetti 1986; Jamieson 1991; Jamieson et al. 1999; Morrow 2004; Pitnick et al. 1995a; Pitnick et al. 2003; Sivinski 1984), as expected of traits subject to intense sexual selection (Andersson 1994; Eberhard 1985). Second, intraspecific variation in sperm size positively correlates with fertilization success in the bulb mite *Rhizoglyphus robini* (Radwan 1996), the nematode *Caenorhabditis elegans* (LaMunyon and Ward 1998) and in the freshwater snail *Viviparus ater* (Oppliger et al. 2003). Third, selection lines of *C. elegans* evolved larger sperm in response to experimentally increased levels of sperm competition (LaMunyon and Ward 2002), and males from lines of the fruitfly, *Drosophila melanogaster*, experimentally selected to have longer sperm demonstrated enhanced competitive fertilization success (Miller & Pitnick 2002; but see Gage & Morrow 2003, discussed in detail below). Fourth, comparative studies of a

diverse array of taxa have found a significant positive relationship between sperm length and the risk or intensity of sperm competition (mammals: (Gomendio and Roldan 1991); primates: (Dixson 1993); birds: (Briskie and Montgomerie 1992; Briskie et al. 1997; Johnson and Briskie 1999); butterflies: (Gage 1994); nematodes: (LaMunyon and Ward 1999); moths: (Morrow and Gage 2000); cichlid fish: (Balshine et al. 2001); frogs: (Byrne et al. 2003); rodents: (Breed 2004); but for exceptions see (Stockley et al. 1997) on fish and Harcourt 1991; Hosken 1997; (Anderson and Dixson 2002; Gage and Freckleton 2003; Harcourt 1991; Hosken 1997)on mammals, discussed in detail below). Fifth, comparative studies on diverse taxa have found significant correlated evolution between sperm length and dimensions of some critical region of the female reproductive tract (featherwing beetles: (Dybas and Dybas 1981); birds: (Briskie and Montgomerie 1993); fruit flies: (Pitnick et al. 1999b; Pitnick et al. 2003); stalk-eyed flies: (Presgraves et al. 1999); moths: (Morrow and Gage 2000); dung flies: (Minder et al. 2005); but see (Hosken 1998) for megachiropteran bats). The interpretation that this correlation results from sperm size evolving in response to changing female reproductive tract design is supported by an experimental evolution study showing that evolving female sperm-storage organ morphology can drive the evolution of sperm length (Miller and Pitnick 2002; Miller and Pitnick 2003). Long flagella, as the overwhelming contributor to overall sperm length, are thus best thought of as ornaments or armaments - the result of postcopulatory sexual selection for traits that enhance competitive fertilization success (Keller and Reeve 1995; Miller and Pitnick 2002). Likewise, the conditions of the female reproductive tract, which bias fertilization success in favor of certain sperm

phenotypes over others, represent the proximate bases of female sperm or sire choice (Pitnick and Brown 2000).

Although there is strong evidence for correlated evolution between certain dimensions of the female reproductive tract and the gamete size being favored (Miller 2002), no mechanisms have yet been proposed to explain this interaction. In a previous study (Pitnick et al. 1999b; Pitnick et al. 2003), sperm in *D. melanogaster* seminal receptacles was observed to exhibit a reproducible pattern of organization that was confirmed and expanded upon in this paper. Both long - and short - seminal receptacle females present an organization pattern consistent with relatively few sperm residing in a mass proximate and highly removed from the majority of sperm in storage in the distal end of the organ. Until now, this is all that was indicated.

The origin of anisogomy is unknown, however disruptive selection acting upon an originally isogamous population is the popular theory to explain its emergence (Bulmer and Parker 2002; Parker et al. 1972) . Sperm competition theory applies the same selective conditions (i.e., the more numerically abundant gamete type competing to fuse with the rarer gamete type) to explain the evolutionary maintenance of anisogamy (Parker 1982). Specifically, most theoretical treatments model sperm competition as a raffle, with the probability of a given male siring an offspring depending on the relative representation of his sperm in the "fertilization set" (Parker 1970b; Parker 1982; Parker 1984a; Parker 1990a; Parker 1990b; Parker et al. 1972; Parker et al. 1996; Parker et al. 1997; Williams et al. 2005). Under these conditions, males will be selected to invest minimally in

each sperm (i.e., tiny sperm) and thus maximize the number of sperm produced (e.g., (Parker 1970b; Parker 1982; Parker 1984a; Parker 1990a; Parker 1990b; Parker et al. 1972).

All other things being equal, greater sperm numbers should nearly always enhance male fertilization success (with the possible exception of species where males can efficiently remove, incapacitate or displace previously stored sperm; e.g., (Waage 1979). This prediction has received robust empirical support. First, experiments with numerous taxa have demonstrated that males copulating longer, transferring larger ejaculates or greater numbers of sperm achieve paternity (Birkhead and Møller 1998a; Simmons 2001). Second, this sperm quantity advantage certainly underlies the taxonomically widespread relationship between relative testis mass and the intensity of sperm competition demonstrated through comparative analyses (e.g., (Harcourt et al. 1981; Pitcher et al. 2005; Ramm et al. 2005). Third, males from populations for which sexual selection has been experimentally eliminated evolve relatively smaller testes (Hosken and Ward 2001; Pitnick et al. 2001). Nevertheless, things are not always equal (Snook 2005). For example, among insects, sperm quality, as measured by sperm viability, positively co-varies with the intensity of sperm competition (Hunter and Birkhead 2002). Also, experiments controlling the number of sperm inseminated into females have found repeatable and/or heritable differences among males in ejaculate performance or the outcome of sperm competition (Birkhead et al. 1999; Dziuk 1996; Froman et al. 2002; Martin et al. 1974).

Theoretical treatments of sperm size evolution have approached the problem from a parental investment theory perspective (exceptions discussed below), with the principal adaptive benefit of larger sperm being enhanced zygote viability (Bulmer and Parker 2002; Parker 1982; Parker 1984a; Parker et al. 1972; Trivers 1972). Such models indicate that, with a starting condition of extreme anisogamy, an increase in sperm size will only be favored when there is no sperm competition. Parker (1982, p. 287) summarizes the conclusion as follows: "*Essentially, the reason it does not pay to increase sperm provisioning is that a unit increase in investment in each sperm causes significant cost, but insignificant benefit. For example, doubling the sperm size halves the sperm number, which causes significant losses when there is sperm competition. But doubling the sperm size would effect a virtually insignificant increase in the viability of the zygote*."

As we have found that large sperm are, in fact, advantageous in certain systems, we contend that parental investment theory provides a limited perspective for considering sperm size evolution. It is true that the entire sperm cell enters the egg in the majority of species (Ankel-Simons and Cummins 1996; Karr and Pitnick 1996; Snook and Karr 1998) and that post-fertilization interaction between the "sperm" and the "egg" can be protracted and complex (Karr 1991; Pitnick and Karr 1998). It is also now recognized that the sperm contributes more essential product to the zygote than simply the haploid complement of paternal DNA (Churchill et al. 2003; Karr 1996; Krawetz 2005; Loppin et al. 2005; Rauh et al. 2005; Schatten 1994). Unfortunately, very little is know about fertilization in

animals other than chordates and echinoderms (Sander 1985), and even less is known about the fate of sperm-derived products following fertilization (Karr 1996; Krawetz 2005; Pitnick and Karr 1998). Nevertheless, the only relevant study to date strongly suggests that post-fertilization function is not the driving force behind evolutionary diversification of sperm size (Karr and Pitnick 1996). Thus, although postzygotic traits were explicitly considered by Trivers (1972) as parental investment, we contend that sperm "quality" attributes arising from postcopulatory sexual selection represent energies expended in intrasexual competition and intersexual choice, and hence are specifically excluded from parental investment by Trivers (1972).

We also have a poor understanding of sperm-female interactions (Pitnick et al. 1999b). We know little about the dynamics of sperm motility inside of females (Katz and Drobins 1990), and hence very little of structure-function relationships for spermatozoa (e.g., (Moore et al. 2002). Likewise, there is no robust understanding of how sperm move or are transported to sites of storage and fertilization for most taxa (e.g., Tschudi-Rein and Benz 1990; (Steele and Wishart 1992; Suarez 2002b; Tschudi-Rein and Benz 1990). We have only meager details for few taxa about how sperm are organized within females (both within and among alternative sperm-storage organs; Siva-Jothy 1987; (Fritz and Turner 2002; Gack and Peschke 1994; Otronen et al. 1997; Siva-Jothy 1987). We know even less about how sperm from different males may interact with one another (Birkhead and Møller 1998b) and of how sperm viability is maintained during prolonged storage (Austin 1975; Davey and Webster 1967; Filosi and Perotti

1975; Foighil 1985; Fritz and Turner 2002; Racey 1979; Suarez 2003; Wheeler and Krutzsch 1994). This lack of knowledge of the selective environment for sperm has likely contributed to little attention being paid to sperm adaptations.

We do know that female reproductive tracts tend to be complex (Birkhead et al. 1993; Eberhard 1996; Keller and Reeve 1995) and that female tract design, especially that of the sperm-storage organs, can be highly evolutionarily divergent (e.g., (Dybas and Dybas 1981; Pitnick et al. 1999b; Pitnick et al. 2003; Siva-Jothy 1987). Anecdotal evidence suggests that in a diversity of taxa, sperm undergo biochemical, morphological and/or behavioral modification within females (e.g., Nur 1962; Makielski 1966; (Bedford and Shalkovsky 1967; Hughes and Kavey 1969; Makielski 1966; Nur 1962; Renieri and Talluri 1974; Rieman and Thorson 1971). In mammals, sperm are held in the oviduct by binding to the surface of the oviductal epithelium, prior to capacitation (Fazeli et al. 1999; Fazeli et al. 2000; Suarez 2002a; Suarez 2003). Sperm-female interactions contributing to differential male fertilization success may be complex (Fazeli et al. 2004; Georgiou et al. 2005) and may include sperm-female-seminal protein interaction effects (Peng et al. 2005). Variation among males in sperm traits that interact with females are likely to contribute to differential male competitive fertilization success (Miller and Pitnick 2002; Peng et al. 2005; Watnick et al. 2003) and, hence, serve as targets for postcopulatory sexual selection.

Demonstrating postcopulatory female choice experimentally is highly challenging, which is why there are a poverty of studies examining this directly.

The challenge is to demonstrate both the capacity of females to bias paternity in favor of one male's sperm over that of another, and to understand how such an ability would be favored over, or in addition to, mechanisms that would act earlier in the course of events, such as pre-copulatory mate choice (Birkhead 1998; Eberhard 1996; Pitnick and Brown 2000; Telford and Jennions 1998). Mechanisms underlying female sperm choice are inherently difficult to study (Birkhead and Pizzari 2002) and hence there have only been a few demonstrations (reviewed in (Birkhead 1998; Pitnick and Brown 2000; Telford and Jennions 1998) see also (Mack et al. 2002; Miller and Pitnick 2002); also see studies of conspecific sperm precedence: Howard 1999; (Eady 2001; Howard 1999). Moreover, the majority of the studies demonstrating female sperm choice reveal biases against the sperm of closely related or otherwise genetically incompatible males, and thus should not contribute to directional selection on sperm traits (Birkhead 1998; Clark et al. 1999; Zeh and Zeh 1997). Only a single study (Miller and Pitnick 2002) identifies interacting male and female traits that connect to a broader macroevolutionary pattern (Pitnick et al. 1999b; Pitnick et al. 2003).

A final theoretical constraint that influences our understanding of sperm quality evolution by sexual selection is a consequence of the centrality of Bateman's (1948) contribution in sexual selection theory. Bateman’s quantitative description of sex differences in *D. melanogaster* gave rise to the modern era of sexual selection theory (Clutton-Brock and Parker 1992; Emlen and Oring 1977; Shuster and Wade 2003; Trivers 1972) by showing that the slope of the line relating

reproductive success to mating success (the sexual selection gradient) is nearly flat for females, whereas the slope of this line is much steeper for males. The magnitude of the sex difference in the strength of selection depends upon the relationship between male and female sexual selection gradients (Jones et al. 2002; Jones et al. 2000). Anisogamy generates the conditions for sexual selection, as numerically abundant male gametes compete to fertilize rare female gametes (Kokko and Jennions 2003). For the majority of species, those lacking post-fertilization parental investment (e.g., most *Drosophila*; (Pitnick et al. 1997), the intensity of sexual selection distills down to the sex difference in the number of gametes produced. Because sperm size and number are expected to trade-off (Oppliger et al. 1998; Pitnick 1996), the evolution of giant sperm by sexual selection is an apparent paradox: as sperm size increases, sperm become less abundant, ova become relatively less rare, and hence competition between sperm (or males) for fertilization success is predicted to weaken. As a consequence, theory predicts an inverse relationship between sperm size and the intensity of sexual selection on sperm quality (Bjork and Pitnick 2006).

What is needed to clarify our understanding of sexual selection for sperm quality, and to recognize that certain sperm characters are secondary sexual traits, is (1) an understanding of the relationship between sperm quality (e.g., size) and the intensity of sexual selection, (2) knowledge of how sperm quality and quantity contribute to the pattern of sperm precedence, (3) elucidation of the mechanisms by which sperm and the female reproductive tract interact to generate selection on sperm quality and (4) identification of the selective benefits accrued by females

from choosing among sperm. By repeating Bateman's (1948) experiments with species of *Drosophila*, as well as with experimental evolution lines of *D. melanogaster* that differ in sperm length (Miller and Pitnick 2002), we have recently made progress toward the first goal by demonstrating that the opportunity for sexual selection does not decrease with increasing sperm length (Bjork and Pitnick 2006). Herein, working with the same lines of *D. melanogaster*, we report the results of experiments fulfilling the second and third goals (but not the fourth goal). Specifically, using a fully factorial design, we investigate the effect of varying sperm length and sperm number on second male sperm precedence. Next, we provide a detailed examination of the distribution of sperm within the primary sperm-storage organ, revealing a pattern of organization that corresponds to the architecture of the female organ. Finally, we quantify the distribution of competing short and long sperm within females to reveal some of the mechanisms by which males with relatively long sperm achieve a fertilization advantage.

Methods

Experimental populations and culturing

All experiments were conducted on populations of *D. melanogaster* artificially selected bi-directionally for either sperm length or seminal receptacle (SR) length. Details of the selection protocols and of the source populations are provided in Miller and Pitnick (2002, 2003). Males were from "short-sperm" or "long-sperm"

populations, 36 -48 generations following the inception of selection on sperm length. Females were from "short- SR" or "long-SR" populations (replicate B), 58-60 generations following the inception of selection on SR length. Note, however, that these populations have not been subject to selection for sperm or SR length since generations 17 and 38, respectively. Nevertheless, as demonstrated by data presented herein, no appreciative regression of the traits has occurred.

Additionally, for the sperm competition experiment, LH_M-*BW* strain males were used. This strain was derived from a large outbred population (LH_M) that had adapted to the laboratory for over 200 generations, and carries a brown-eyed (*BW*) dominant marker that had been introgressed through 12-13 back-cross generations into the LH_M background (see (Chippindale et al. 2001) for details on the origin and maintenance of these lines). These lines were obtained from A. Chippindale and maintained in our laboratory since their arrival in 2001 in a population cage supporting > 1000 individuals with overlapping generations.

All flies were reared at moderate density on standard cornmeal molasses agar medium at 25° C and a 12L:12D cycle. Males and females were collected from culture bottles as virgins following light ether anesthesia and stored 10 flies per 8-dram vial with medium inoculated with live yeast until reaching experimental age.

Sperm and SR dimensions

For some experimental analyses, sperm length and SR length were treated as discrete factors (e.g., long- versus short-sperm line). In other cases, it was necessary to measure mean sperm head or total length for individual males and SR length for individual females. Sperm of each anesthetized male were measured following dissection of the seminal vesicles into phosphate-buffered saline (PBS) on a subbed slide. After passively releasing a few hundred sperm into the saline, preparations were dried in a 60° C oven, fixed in methanol:acetic acid (3:1), stained in a 5×10^{-7} M solution of Hoechst 33258 (Sakaluk and O'Day 1984) and then mounted with glycerol:PBS (9:1) under a glass coverslip. Digital images of sperm were obtained using a Dage CCD72 camera (Dage-MTI Inc., Michigan City, IN, USA) mounted on an Olympus BX60 microscope (Olympus America Inc., Melville, NY, USA) and lengths were measured using NIH Image public domain software (http://rsb.info.nih.gov/nih-image). Total sperm length was quantified using darkfield optics at a magnification of 200X and sperm head length using epifluorescence at 1000X.

Prior to examining the mechanisms conferring a fertilization advantage to relatively long sperm, it was necessary to discern (1) population (selection line) differences in the mean and variance of sperm length, (2) the relationship between sperm head length and total length. We thus measured both head and total length for each of 20 sperm per male (N = 15 males per line). These data confirmed that within-male variation in sperm sperm length was low (Fig. 1), that the long- and short-sperm lines exhibit non-overlapping distributions in total sperm length (Fig. 2) and that these populations also differ significantly in the length of sperm heads (Fig. 2). Thus, these lines could be experimentally used to explore the

contribution of sperm quality and quantity to differential male fertilization success and the mechanisms underlying the demonstrated advantage of relatively long sperm (Miller and Pitnick 2002).

SR length was determined for each anesthetized female by dissecting the reproductive tract into PBS on a microscope slide, paring away extraneous tissue with fine probes, and severing the tracheoles binding together the loops of the SR. A glass coverslip with clay at the corners was then placed on top of the specimen, and the clay was carefully compressed, while viewing through a microscope, until the SR was flattened to two dimensions, but without over-compressing and thus stretching the organ. The preparation was then viewed and a digitized image captured at 200X using differential interference contrast microscopy. Using NIH Image, diameter of the SR lumen was measured approximately every 0.10 mm and SR length determined by tracing the lumen from proximal to distal ends.

Contribution of sperm quality and quantity to competitive fertilization success

The contributions of sperm quality and quantity to male competitive fertilization success were determined by assaying second male sperm precedence (P2, arcsine square root transformed) while factorially varying the quality (short versus long) and quantity (few versus many) of sperm transferred by second males. All females were initially mated to an LH_M-*BW* male and then remated after three days to a wild type (long- or short-sperm selection line) male transferring either (1) many long sperm, (2) few long sperm, (3) many short sperm, or (4) few short

sperm (N = 20 per treatment), with females randomly assigned to second-male treatments. Sperm quantity was manipulated by varying the number of copulations performed by the male prior to the experimental copulation (Fig. 3). Only females from the long-SR selection line were used for this experiment, as these demonstrate the greatest level of sperm choice in favor of longer sperm (Miller and Pitnick 2002).

The general design of the experiment was identical to that used by Miller and Pitnick (2002). Virgin 4-6 day-old females were initially mated and then remated to an experimental male 3 days later. Females were transferred to fresh vials containing media and live yeast immediately following remating. They remained in these vials for 24 h and were then transferred to a second vial for 24 h before being discarded. After all progeny had eclosed, paternity was ascertained by eye color and P2 was calculated as the proportion of offspring sired by the second male. The number of progeny eclosing from vials occupied by each female prior to remating was quantified and this variable (an index of the number of first male sperm used by the female prior to remating) was entered as a continuous covariate in the statistical analysis of P2.

Two preliminary experiments were conducted to determine the appropriate number of prior matings to subject long- and short-sperm males to manipulate sperm quantity. The number of sperm transferred by males was assayed directly in one experiment by counting the number of sperm ejaculated into each of five successive control-line females (N = 5 males per line; Fig. 3A) and indirectly in a

separate experiment by counting the number of progeny produced by six successive control-line mates (N = 20 males per line; Fig. 3B). In both experiments, each male was paired with a virgin female and transferred to a vial containing a new virgin female immediately following termination of each successive copulation. For sperm transfer, females were frozen immediately following male dismount and were later thawed and the sperm were dissected into phosphate-buffered saline (PBS) from the bursa copulatrix (aka. uterus), seminal receptacle and paired spermathecae (the vast majority of sperm were in the bursa), dried, fixed, stained and then counted under epifluorescence microscopy at 400X. For progeny production, each female was initially retained in the vial in which mating took place, transferred to a fresh vials on days 2, 4 and 6 henceforth, and discarded on day 10. All progeny eclosing from these vials was quantified.

It was important to confirm that long-sperm males, in both many and few sperm treatments, transferred no more sperm than short-sperm males. Otherwise, a statistically significant effect of the factor "sperm length" could arise but in fact be attributable only to a sperm quantity effect. In order to avoid comparing the fertilization success of "virgin" males with that of previously mated males, all males inseminated at least one female prior to being used in an experimental mating. Long- and short-sperm line males in the "many sperm" treatments mated twice or once, respectively, prior to the experimental mating (Fig. 3A). Long- and short-sperm line males in the "few sperm" treatments mated four or five times, respectively, prior to the experimental mating (Fig. 3A). This protocol was conservative in that any probable asymmetry in the number of sperm numbers

transferred, between lines within either the “many sperm” or “few sperm” treatment categories, was biased in the direction of long-sperm line males transferring fewer sperm than did short-sperm line males (Fig. 3A).

Organization of sperm within females

We quantified how sperm from long-sperm and short-sperm line males became organized within the seminal receptacle of twice-mated females through two separate experiments. First, the general organization of sperm throughout the SR, independent of line of sperm origin, was established by “mapping” the position of every sperm within the seminal receptacle in vivo (N = 20 females evenly distributed across two female treatments [short-SR and long-SR selection lines] by two male order treatments [long-sperm line male first and short-sperm line male second and vice-versa]), using the identical protocols and timing of assay to that used in the sperm precedence experiment described above (and used in Miller and Pitnick 2002). Twenty-four hours following remating, females were flash frozen in liquid nitrogen and then frozen to the surface of media at -20° C until dissection. The SR of females was later dissected out, fixed and stained with 2% orcein in 60% acetic acid (Gilbert 1981; Gilbert et al. 1981). The absolute number of stained sperm heads residing within each consecutive 0.10 mm long section of the SR were counted across the entire organ at 400 X using differential interference contrast microscopy (Fig. 5). The diameter of the SR lumen was also measured every 0.10 mm (N = 10 females per line) to assess any morphological variation co-varying with the pattern of sperm distribution (Figs. 5 & 6). This experiment established that sperm adopt a non-random, bimodal spatial frequency

distribution across the proximal and distal regions of the SR (Figs. 5 & 6).

In the second experiment, each female was mated to one long-sperm and one short-sperm line male, and distribution of sperm from the two competing males within each female's SR was quantified by estimating the proportion of both sperm types within the proximal SR and distal SR sperm "sub-populations." This experiment used the identical four mating treatments (N = 30 females per treatment), protocols and timing of assay described above for the sperm organization and sperm competition experiments. Again, females were frozen in liquid nitrogen after 24 h and then frozen to the surface of media at –20° C to await dissection. The SR of these females was later dissected into PBS containing 0.10 % Triton-x. A dissection technique was employed that results in removal of all sperm from the SR as a single, intact, rope-like mass without altering the relative position of sperm within the mass (Fig. 7). These preparations were dried, fixed, stained and mounted. Under these conditions, it was not possible to measure the total length of individual sperm. However, the length of sperm heads could be accurately measured, adn this was done under epifluorescence at 1000X as described above. For each female, the heads of all sperm occupying the proximal end of each SR were measured, as were a random sample of 100 sperm occupying the distal end of the SR. Due to the challenging nature of the dissection technique, not all dissections were successful and hence final sample sizes of treatments vary (N = 19–29).

Because the respective distributions of sperm head lengths for the long- and short-sperm lines overlap (Fig. 2), we used an EM (Expectation Maximization) algorithm to estimate the proportions of the two sperm categories (long and short) in the observed mixed distributions (Hasselblad 1966; Ott 1979). The algorithm is implemented in the program NOCOM available from ftp://linkage.rockefeller.edu/software/utilities/.

In order to estimate the proportions we determined the means and variances of the two categories to be used in the algorithm. Estimates of the variances of each category were obtained from earlier observations on sperm lengths in non-mixed distributions (data illustrated in Fig. 2). These were found to be similar and estimated to be $S^2 = 0.25$. The estimates for the two means were obtained from decomposition of the overall data (n = 12,181) using a known common standard deviation (0.5), and unknown proportions (p_1, p_2). The two means were estimated to be $\hat{u}_1 = 9.21$ and $\hat{u}_2 = 10.14$. Throughout the analysis we used these conditions ($\hat{u}_1 = 9.21$, $\hat{u}_2 = 10.14$, common S = 0.5) to estimate proportions of the two sperm types in the distal/proximal parts of the SR of (1) each female, and (2) females pooled over each treatment category. Note, however, that when neither means nor proportions were provided, such that both had to be estimated by the NOCOM program, the resulting estimated proportions were nearly identical to those presented.

To evaluate the validity of the mixed-distribution model (two component) as compared to a model based on one component ($u_1 = u_2$, $\sigma = 0.5$) we used a

likelihood-ratio (LR) test of the hypothesis that the one-component model provides the same fit as the two-component model, i.e., $LR = -2(L_1 - L_2)$ where L_1 and L_2 are the log-likelihoods of the one-component and two-component models respectively. In this case the LR statistic is distributed as a X^2 with 2 degrees of freedom when the number of observations are large (Thode et al. 1988). We calculated the LR statistic for the pooled data in each of the four treatment categories and found that all showed significant improvement of fit using the two-component model ($P < 0.001$).

We further assessed the efficacy of the decomposition algorithm for estimating the proportions of two sperm populations within sperm mixtures by conducting a simulation experiment. Empirical observations of sperm head lengths for the long- and short-sperm lines (Fig. 2; long sperm n = 279, short sperm n = 265) were used to create a series of mixed distributions of known proportion of the two sperm types. Each mixed distribution had a sample size of n = 50, corresponding to the approximate minimum numbers counted in samples from the proximate end of the SR of individual females. In the simulation, the proportion (p) of the long sperm type (1-p for the short sperm type) was specified as 0.l0, 0.30, 0.50, 0.70 or 0.90. A random number generator was used to select $n_1 = p*n$ long sperm and $n_2 = (1-p)*n$ short from the empirical data sets; thus, the proportion was known for the mixing process. The proportion of long sperm in the simulated mixed distributions were then estimated using the EM algorithm. Only p was estimated using the original head length means of the long and short sperm (10.67 and 9.73

μm, respectively) and a common variance of 0.25. For each specified p, 20 females (each with 50 sperm) were used to estimate 20 p values Table 1.

Note that the bias in the estimate at p = 0.10 (in particular) was due to the variance of the short-sperm class in the sample being higher than the value of 0.25 used in the model, whereas the long-sperm class had a variance of 0.25. Because the recommendation for use of the EM algorithm (Hasselblad 1966; Ott 1979) is to use a common variance, we chose to use the smaller of the two empirically determined values, as additional simulations showed this approach to be conservative, with higher variances resulting in greater proportional representation by the longer sperm class.

Results

Variation in sperm and SR dimension

There was relatively little within-male variation in total sperm length in the selection lines. An analysis of mean male sperm length based on 20 sperm per male and 15 males per line for the combined long-sperm and short-sperm lines revealed that measuring only a single sperm captures 80.4% of the variation in sperm length within males. Means based on measures of two sperm per male captures 91.2% of the variation, and the number of sperm required to estimate mean sperm length asymptotes at 4 sperm, with 96.1% of the variation captured (Fig. 1).

The total length of sperm differed significantly between the long-sperm and short-sperm selection lines ($F = 5356.08$, $P < 0.0001$, $n_{long} = 278$, $n_{short} = 265$) and exhibited non-overlapping distributions (Fig. 2). Sperm head length similarly differed significantly between the two lines ($F = 340.93$, $P < 0.0001$, $n_{long} = 278$, $n_{short} = 265$), although the distributions largely overlap (Fig. 2). A regression analysis of sperm head length on total length using all data from both the long-sperm and short-sperm lines results in a highly significant relationship between these two characters ($R^2 = 0.348$, $F_{1, 541} = 289.28$, $P < 0.0001$). However, performing the analysis separately by line reveals no significant relationship between sperm head and total length within either the long-sperm line ($R^2 = 0.0000$, $F_{1, 276} = 0.001$, $P = 0.973$) or the short-sperm line ($R^2 = 0.0004$, $F_{1, 263} = 0.119$, $P = 0.731$) (Fig. 2).

The total length of the female's SR also differed significantly between the long-SR and short-SR selection lines (Fig. 6), exhibiting non-overlapping distributions. The SR was found to be a heterogeneous structure, as the diameter of the organ's lumen varied across its length. The lumen of the organ at its entrance, where it emanates from the anterior-ventral bursa, is relatively wide, with an inner diameter of approximately 27 μm. The lumen in this region appears funnel-like, rapidly narrowing to approximately 7 μm over the proximal 0.3 mm of organ length. The inner diameter of the lumen remains this narrow for approximately 1.1 mm and 1.4 mm in the short-SR line and long-SR line females, respectively. At this point, the inner diameter of the lumen abruptly widens and remains 20 - 25

μm wide throughout the distal region of the SR, before tapering down to 13 μm at the organ's terminus (Figs. 5 & 6).

Contribution of sperm quality and quantity to competitive fertilization success

Both sperm quality (i.e., length) and sperm quantity contributed significantly to male competitive fertilization success (Table 2). Specifically, both longer sperm and greater numbers of sperm independently contributed to increased male competitive fertilization success. These results thus replicate the sperm quality advantage reported by Miller & Pitnick (2002). There were also three significant interactions: "sperm length x sperm number," "sperm length x prior progeny" and "sperm length x sperm number x prior progeny." We evaluated the slopes of the interaction terms and determined that none influenced the interpretation of the main effects. The significant "sperm length x sperm number" interaction is of particular interest, as it indicates that the advantage in sperm competition afforded by sperm quality increases as the number of sperm competing declines (Table 2; Fig. 4).

Organization of sperm within females

The distribution of sperm throughout the SR was found to be heterogeneous. There was a spatially bimodal distribution of sperm heads with relatively few heads clustered in the proximate (0.5 mm) end of the organ, followed by a roughly 1.0 mm long section containing virtually no sperm heads, and finally a

great many sperm heads distributed throughout the distal end (approximately 40%) of the organ (Fig. 6). The central region lacking sperm heads was not void of sperm, but rather was occupied by the tails of the sperm heads residing in the proximal end of the SR. The transition in the SR from the lumen containing only the flagella of the proximate "cohort" of sperm heads to it containing a great many sperm heads in the distal region is coincident, in both short-SR and long-SR lines, where an abrupt widening of the lumen by approximately four times occurs (described above; Figs. 5 & 6).

Analysis of variance (ANOVA) treating female line (long-SR or short-SR) and male mating order (long-sperm line male first/short-sperm line male second or vice versa) revealed highly significant effects of female line on the total number of sperm stored in the SR ($F_{1, 17} = 83.71$, $P < 0.0001$; mean ± se: long-SR line: 200 ± 7, short-SR line: 117 ± 6), as well as in the number of sperm occupying both the proximate ($F_{1, 17} = 105.95$, $P < 0.0001$; mean ± se: long-SR line: 52 ± 3, short-SR line: 26 ± 1) and the distal regions ($F_{1, 17} = 42.87$, $P < 0.0001$; mean ± se: long-SR line: 148 ± 8, short-SR line: 91 ± 6). In all categories, long-SR line females stored more sperm than did short-SR line females (Fig. 6), confirming the report by Miller & Pitnick (2003).

There was no significant effect of male mating order on either the total number of sperm stored ($F_{1, 17} = 2.00$, $P = 0.18$) or on the number of sperm in the distal region of the SR ($F_{1, 17} = 0.45$, $P = 0.51$). There was, however, a significant effect of male order on the number of sperm stored in the proximal region of the SR ($F_{1, 17} =$

7.38, P = 0.015), with females from both lines storing more sperm in the proximal region when their second mate was a long-sperm line male (Fig. 6). There were no significant female line by male mating order interaction effects.

Following the discovery that sperm within the SR are spatially organized into two discrete populations: proximal and distal (Fig. 6), we investigated the contribution of short and long sperm to each of these populations in twice mated females. Using the same four mating treatments described immediately above, sperm were dissected from the SR and the heads of all sperm occupying the proximal end were measured, as were a haphazard sample of sperm heads from the distal region of the SR. These observed mixed distributions of sperm head length data were decomposed using the EM algorithm to estimate the proportions of long and short sperm in three sequential analyses. First, all sperm head length measures from both regions of the SR from all females and all four treatments were combined prior to decomposition in order to estimate the proportions of long and short sperm that were stored by females. Second, the four mating treatments were analyzed separately, yet within each treatment all sperm head length data from the proximal and distal regions were respectively combined for decomposition analysis. Third, the proportions of long and short sperm found in the proximal and distal regions of the SR were uniquely estimated for each experimental female.

The experiment-wide analysis of all sperm measured generated estimated proportions of 0.32 and 0.68 for the short and long sperm, respectively. Thus,

despite each female having been inseminated by one short-sperm line and one long-sperm line male, with both mating orders equally represented, approximately twice as many sperm from long-sperm line males was found to reside within the SR of females. Although these males inseminate more sperm than do short-sperm line males (Fig. 3A, first mating), this difference is not significant ($F_{1, 8} = 2.50$, P = 0.153, N = 10; mean ± se: short-sperm line: 2375.4 ± 95.2; long-sperm line: 2553.8 ± 60.6), and could not account for the disparity in number of sperm stored.

In the next analysis, which discriminated among treatments and proximal and distal regions of the SR but combined data for all females with treatments, the proximal region of the SR was estimated to comprise 80 – 94% long sperm and the distal region 45 – 78% long sperm (Table 3). Not surprisingly, given the well-established pattern of second-male sperm precedence in *D. melanogaster*, long-sperm biased proportions were higher when the long-sperm line male was the second mate. It is a striking, however, that short-sperm line males do not achieve greater than 55% representation in the distal region of the SR, even when mating second, and they never achieve higher than 20% representation in the proximal region of the SR (Table 3).

In the analyses conducted on a per female basis, estimated proportions of long and short sperm in the proximate and distal regions of the SR reveal an extreme bias in the pattern of sperm storage. Across all four treatments, mean sperm head lengths were consistently longer in the proximal versus the distal region of the SR (Table 4; Fig. 8). Irrespective of mating order, the sperm of long-sperm line

males never contributes less than 60% on average to the sperm present in the distal end of the SR, and never less than 83% on average to the sperm in the proximate end of the SR (Table 4). The difference in the representation of both sperm categories between the proximal and distal regions was highly significant ($P < 0.0001$) in all treatments (Table 4). The greatest disparity (22 - 23% difference on average) between the proximal and distal ends of the SR in the proportional representation of sperm was observed in the two treatments with short-sperm line males mating second. In these two treatments, long sperm accounted for 60 – 67% on average of the sperm present in the distal end of the SR, yet accounted for 83 – 90% on average of the sperm in the proximal end (Table 4). An ANOVA testing the difference between the proximal and distal regions of the SR in the proportion of long sperm ($N = 93$) found no significant effect of female line ($F_{1, 89} = 0.30$, $P = 0.5834$), but significant effects of both male mating order ($F_{1, 89} = 10.09$, $P = 0.0021$) and the female line by male mating order interaction effect ($F_{1, 89} = 4.77$, $P = 0.0315$).

Discussion

Mechanisms of Sperm-female Interaction

Sperm quality (i.e., length) significantly contributed to male fertilization success (Fig. 4, Table 2). This result confirms the findings of Miller & Pitnick (2002) and supports the conclusion that the relatively long sperm flagella of some *Drosophila* species are the product of sexual selection (Karr and Pitnick 1996; Miller and Pitnick 2002; Pitnick et al. 1999a; Pitnick and Markow 1994). Miller & Pitnick

(2002, 2003) postulated that sperm quality attributes were likely to coevolve with female reproductive tract design, and supported this contention using experimental evolution techniques to reveal significant sperm morphology by female reproductive tract morphology interactions on male competitive fertilization success. Here we identify likely mechanisms underlying this sperm-female interaction, thus revealing the means by which female tract design generates sexual selection on sperm design.

The seminal receptacle is the only female sperm-storage organ of many *Drosophila* species and, for those species utilizing both the SR and the spermathecae, the SR is believed to be the primary reservoir of sperm used for fertilization (Pitnick et al. 1999a). When an egg descends the common oviduct and enters the bursa to await fertilization, the anterior egg pole with its micropyle (the tube through which the fertilizing sperm must travel) occupies a "fertilization chamber" at the orifice of the SR (see Figure 1 of (Sander 1985). It is reasonable therefore to assume that sperm occupying the proximal end of the SR are better positioned to compete for access to the egg micropyle than are sperm more distally located in the organ, and hence take precedence over them. It is thus relevant that the lumen of the SR was found to be heterogeneous across the length of the organ, being narrow throughout the proximal end and wide in the distal end. This morphology was coincident with a nonrandom distribution of sperm within the organ. Two discrete subpopulations of sperm were found in the SR of all females: a relatively small and well-organized group in the proximal half of

the organ and a larger and more haphazardly organized group in the distal half (Figs. 5 and 6).

Two putative mechanisms by which longer sperm achieve a fertilization advantage were identified. First, irrespective of mating order, longer sperm were more likely to be stored in the SR than were shorter sperm. Long-sperm line males contributed over 60% of the sperm in the SR on average when they were first mates, and over 90% on average when they were second mates (Table 4). This effect may be attributed in part to long-sperm line males transferring more sperm per ejaculate than short-sperm lines males (see Fig. 3, mating sequence = 1). However, the male line difference in number of sperm transferred was not statistically significant (long-sperm line: 2553.8 ± 60.6; short-sperm line: 2375.4 ± 95.2; $F = 2.498$, $N = 10$, $P = 0.153$), and so is unlikely to explain the dramatic sperm length effect. Consistent with this biased proportional representation of longer sperm, a greater absolute number of sperm was found in the SR when long-sperm line males mated second (compare black with white bars in Fig. 6). Thus longer sperm are better at occupying and/or retaining their occupancy in the SR than are shorter sperm. Second, with regard to occupancy in the proximal region of the SR, longer sperm are better at displacing shorter sperm, and better at resisting being displaced by shorter sperm (Tables 3 and 4; Fig. 8). We do not know how having a longer flagellum confers these storage advantages to sperm.

In an earlier report (Miller & Pitnick 2002), the fertilization advantage of longer sperm was observed in long-SR line females only, whereas the distributional

effects of sperm length were observed here in both short-SR and long-SR line females (Tables 3 and 4; Fig. 8). We can only speculate on the basis for the different results. It is our strong suspicion that previously observed favoritism by long-SR females for longer sperm that was not demonstrated in females with short receptacles is likely attributable to the inability of short-SR females to store many sperm proximally, coupled with the immense overlap in sperm length present in the earlier study. Previously, sperm selection lines could not be directly competed against one another *in situ*, as no mechanism for identifying sperm type within the female was developed, and offspring were indistinguishable. As a result, previous studies (Miller and Pitnick 2002) competed selection line males against LH_M-*BW* strain males carrying a brown-eyed (*BW)* marker in order to assign paternity to resulting progeny. It was our discovery that sperm head length alone could be used to assign sperm paternity within the SR that allowed us to directly compete males from differing selection lines in this study. As sperm selection lines were therefore competed against each other only indirectly in the past (and directly against a control male with high degree of sperm length overlap), it is possible that even short-SR females are capable of effecting a biased distribution in the current study, given the more extreme disparity in sperm length between competitor males.

In conceptualizing the results presented here, one could classify the mechanisms examined as male-mediated or female-mediated, and to attribute them to either sperm competition or cryptic female choice (or more specifically, female sperm choice). We suggest, however, that such definitions are not meaningful as there is

a single continuum defining male- and female-mediated processes influencing postcopulatory reproductive success (Eberhard 1998; Eberhard 2000). At one end of this continuum, there is sperm competition in its most narrow sense, with exploitation competition to fertilize eggs or interference competition among sperm (see (Baker and Bellis 1987), but note that such interference competition among sperm has never been demonstrated, e.g., (Moore et al. 1999), and no interaction with, or fertilization bias generated by, the female reproductive tract. In this scenario, females are passive vessels in which sperm competition takes place. Such conditions, however, may only be met outside of females, in externally fertilizing species. On the cryptic female choice end of the continuum are mechanisms such as sperm ejection by females (e.g., (Pizzari and Birkhead 2000). In most instances, it will not be possible to discriminate male- from female-mediation in the evolution of sperm traits. As argued by Eberhard (Eberhard 1996; Eberhard 1998), female morphology, physiology and behavior determine the playing field and the rules of the game by which males compete. In *Drosophila*, for example, there may be raffle-like exploitation competition among sperm from different males to fertilize ova, with relatively long sperm at an advantage due to selective bias generated by female reproductive tract morphology (among a host of other factors). Hence, male-by-female interactions are expected to explain a significant amount of the variation in fertilization success (Arthur et al. 1998; Clark et al. 1999; Miller and Pitnick 2002; Otronen et al. 1997).

It should be noted that mechanisms of sperm precedence examined here in no way preclude the existence of additional factors contributing to differential male fertilization success in *D. melanogaster*. Consistent with our results, numerous reports have suggested that rival sperm displace resident sperm within the female, although this process had not previously been directly observed (Gilchrist and Partridge 1995; Gilchrist and Partridge 2000; Gromko et al. 1984; Lefevre and Jonsson 1962; Price et al. 1999; Scott and Richmond 1990). Non-sperm seminal proteins (i.e., Acps) are also known to mediate the fate of sperm within females (Wolfner 1997) and hence are likely candidates to mediate sperm competition (Chapman 2001; Chapman et al. 2000). Acps have been experimentally implicated in sperm incapacitation, but the experimental tests of such effect have been indirect and the evidence is thus generally unconvincing (Civetta 1999; Clark et al. 1995; Harshman and Prout 1994; Price et al. 1999). Moreover, one claim of having demonstrated sperm incapacitation (Price et al. 1999) was not repeatable by another laboratory (P. Mack, personal communication), and direct tests of sperm incapacitation in *Drosophila* (Snook and Hosken 2004) and in humans (Moore et al. 1999) suggest that seminal fluids do not kill rival sperm. Rather, it appears for *Drosophila* that loss of resident sperm is the result of females releasing stored sperm from the SR after copulation with a second male (Snook and Hosken 2004).

Sperm Quality and Quantity Effects on P2

Results of the P2 experiment in which both sperm quality and quantity were independently manipulated indicate that both ejaculate attributes independently

influence the pattern of second male sperm precedence in *D. melanogaster* (Table 2, Fig. 4). The preliminary experiment assaying the number of sperm transferred by males from the two lines indicate that the significant sperm quality effect on P2 is unlikely attributable to males from the long-sperm line having transferred greater numbers of sperm. In fact, the test was conservative in that long sperm-line males are estimated to have transferred fewer sperm than did short sperm-line males (Fig. 3A). The magnitude of the sperm quality effect on P2 (Table 2) is striking, especially when considering that the sperm quality disparity between treatments was small relative to the sperm quantity disparity. Long-sperm line males produce sperm that are approximately 28% longer than the sperm of short-sperm line males, whereas males from the "many sperm" treatments are estimated to have transferred 362% more sperm than did males from the "few sperm" treatments.

The major disparity observed between mating treatments was the sheer difference in numbers of stored proximate sperm. Long-SR females were shown to store nearly twice as many sperm proximately on average (Figure 6), and this was verified by our observations of intact receptacles from each treatment in the SR orientation experiment. Comparing this data with the quantity v. quality progeny data, we are left to explain the relatively high P2 exhibited by short-sperm males in the progeny study when examination of sperm storage indicates that Short-sperm males are at a severe disadvantage, even when they are the second male to mate. Since paternity was impossible to assign if long-sperm males were to be directly competed against short-sperm males, LH_M-*BW* dominants that had not

been selected for sperm length were used as a baseline for the progeny study, as in Miller and Pitnick 2002 . We can infer that the degree of overlap between sperm types was great enough to allow short sperm a fair chance at fertilization when competed against 'average' males. However as shown above, Short-sperm males still experienced significantly lower P2 than their long counterparts in both 'many' and 'few' treatments.

There was also a significant sperm length by sperm number interaction effect on P2 (Table 2) that is attributable to the sperm length effect being greater in magnitude when few sperm were competing than when many sperm were competing (Fig. 4). This result suggests that selection on sperm size will have a positive, self-reinforcing momentum. To the extent that sperm quality trades off with sperm quantity (Oppliger et al. 1998; Pitnick 1996), as a lineage responds directionally to selection for increased sperm quality, the strength of selection will intensify as sperm quantity declines, resulting in species for which males produce relatively few gigantic sperm (Bjork and Pitnick 2006). This interaction may in part explain why, contrary to theory based on "Bateman gradients", the "opportunity for sexual selection" (Shuster and Wade 2003; Wade 1979; Wade and Arnold 1980) does not decline with increasing sperm length (Bjork and Pitnick 2006).

Sperm numbers are predicted by theory to be important to male competitive fertilization success (Parker 1984b; Parker 1998), and empirically demonstrated to be important here and elsewhere (Birkhead and Møller 1998a; Simmons 2001).

Nevertheless, results presented here indicate that, for *D. melanogaster*, sperm quality to a certain extent evolutionarily trumps sperm quantity. Large sperm have significant costs associated with their production (Pitnick 1994), including a reduction in the number of sperm produced (Pitnick 1996), the need for relatively large testes (Pitnick 1996) and delayed male reproductive maturity (Pitnick et al. 1995a; Pitnick et al. 1995b). For species with giant sperm, the reduction in the number of sperm produced by each male, the increased metabolic cost of growing and maintaining larger testes (Pitnick 1996) and the protracted age at first reproduction in males relative to females, can in extreme environmental circumstances result in sperm limitation within populations (Pitnick 1993; Pitnick and Markow 1994). It has thus remained an outstanding question for such species as to why hypothetical males that mature rapidly and produce many tiny sperm would not have a fitness advantage. The present study suggest that, due to biases imposed by the design of the female reproductive tract, the numerous sperm of such males would be unlikely to enter the population of sperm that have a chance at fertilization, i.e. the proximate population.

Males of internally fertilizing species do not ejaculate directly onto eggs. In fact, female reproductive physiology has evolved complex mechanisms to control the process of fertilization (Birkhead et al. 1993; Eberhard 1996; Eberhard 1998; Walker 1980). For many species this includes specialized sperm-storage organs (e.g., (Pitnick et al. 1999a). As a consequence, sperm-female interactions can be multifarious, complex and protracted, and may include biochemical, physiological, morphological and behavioral adaptations of both the female and

of sperm (Sivinski 1984). Presumably this is why fish sperm are larger in internally fertilizing than in externally fertilizing species (Stockley et al. 1997), and why, throughout the animal kingdom, there is a general evolutionary pattern of sperm becoming more complex with the origin of internal fertilization (Baccetti 1986). It therefore seems unlikely that only sperm numbers should be subject to postcopulatory sexual selection.

Most theoretical treatments have modeled sperm competition as either a "fair raffle" with the probability of a given male siring an offspring dependent only upon the proportional representation of his sperm in the female (Parker 1970a; Parker 1982; Parker 1984b; Parker 1990a; Parker 1990b; Parker et al. 1972; Parker et al. 1996; Parker et al. 1997), or as a "loaded raffle" with the sperm from the second of two males competitively weighted as a function of the sperm precedence pattern, but otherwise having fertilization success influenced only by sperm numbers (Parker 1990a; Parker et al. 1997). Two models have made fertilization success dependent both on the size and number of competing sperm. In each, the competitive weight of a sperm increases with its size, and size and number trade off, either immediately or over evolutionary time. Sperm size is set by the marginal value theorem and is independent of sperm competition risk (Parker 1993; Parker and Begon 1993). With diploid control of sperm size, the analysis (Parker 1993) suggests that increased sperm size will evolve only when the competitive benefits of size become more important as sperm numbers increase or when sperm size correlates positively with sperm longevity. Predicting the evolutionary response in sperm size is more difficult when sperm

size is under haploid control (Parker and Begon 1993). A final model examines male ejaculate allocation when females exercise sperm choice (Ball and Parker 2003). However, female choice was defined only as a general discrimination of "favorable" or "unfavorable" ejaculates as a reflection of male quality, and thus is not relevant to consideration of sperm form evolution.

Exceptions and Unknowns

Of the numerous comparative analyses that have examined the relationship between sperm size and the risk of sperm competition (see Introduction), five studies have failed to find a significant positive relationship. One of these studies was of fish (Stockley et al. 1997) and the remaining four were of mammals (Anderson and Dixson 2002; Gage and Freckleton 2003; Harcourt 1991; Hosken 1997). These findings too, however, are perhaps consistent with the conclusions of this report, given that most of the fish species included in the analysis have external fertilization, and mammals are unusual in lacking specialized organs and (in most cases) the capacity for prolonged sperm storage by females. With these conditions, the timing of sperm release during a spawn in fish or of insemination relative to ovulation in mammals (Ginsberg and Huck 1989; Huck et al. 1989) and the number of sperm transferred may be the most important attributes conferring fertilization success upon males. Although longer sperm tails are expected to generate greater propulsive force and hence swim faster (Cardullo and Balta 1991; Dresdner and Katz 1981), the dynamics of motility can differ within the ovarian fluid of a spawn in fish (Turner and Montgomerie 2002) and are expected

to be complex and are virtually unknown within female reproductive tracts (Woolley 2003). Moreover, sperm longevity may be an important contributor to fertilization success in fish and mammals. No relationship between sperm length and the longevity of motility was found in a study of Atlantic salmon (Gage et al. 1998). However, this association has not yet received adequate testing (Morrow and Gage 2001b). The more probing question may be why a positive relationship between sperm size and sperm competition was found in another study of fish (but limited to cichlids; (Balshine et al. 2001)) and in a study of frogs (Byrne et al. 2003), which also predominantly have external fertilization.

It must also be noted that lack of a positive relationship between sperm size and the risk or intensity of sperm competition in comparative studies provides at most only weak evidence against the hypothesis that larger sperm are more competitive. In zebra finches, sperm flagellum length exhibits a negative genetic correlation with the length of the midpiece, which also contributes to sperm performance (Birkhead et al. 2005). As discussed above, sperm size has also been demonstrated to trade off with sperm number (Oppliger et al. 1998; Pitnick 1996) and with life history characteristics important to fitness (Pitnick 1996; Pitnick et al. 1995a). The balance of selection on complex male phenotypes in a lineage may not favor larger sperm, but this may not mean that, all other things being equal, males producing relatively long sperm would not accrue a competitive fertilization success advantage. Our understanding of net selection on sperm traits is further complicated by issues of possible sex-biased inheritance (Birkhead et al. 2005; Pizzari and Birkhead 2002).

Careful consideration must be given to another exception to the findings presented here and in Miller and Pitnick (2002). Morrow and Gage (Morrow and Gage 2001a) conducted similar experimental evolution studies of sperm length with the cricket, *Gryllus bimaculatus* (which has ~1 mm long sperm). After five generations of bidirectional selection on sperm length, long-sperm, short-sperm and medium-sperm (control) line males were competed against one another. In contrast to our results, altering sperm length in this cricket elicited no correlated response in sperm competitiveness (Morrow and Gage 2001b). In a follow-up sperm competition experiment with these selected populations (albeit no further selection beyond the initial five generations), paternity success was assayed relative to continuous variation in sperm length and sperm number among competing pairs of males (Gage and Morrow 2003). In striking contrast to our results, along with a significant positive relationship between sperm number and fertilization success, there was a significant negative relationship between sperm length and fertilization success (partial correlations were conducted to control for any covariance between sperm length and number). There were two differences between the *Drosophila* and *Gryllus* projects that may have contributed to the contrasting results. First, although both selection programs produced non-overlapping sperm length distributions between experimental populations, the extent of this divergence was greater in the *Drosophila* study (28% versus 4.5%). Second, only sperm length was experimentally manipulated in the *Gryllus* study, whereas both sperm length and the interacting component of the female reproductive tract were manipulated in the *Drosophila* study. To the extent that

male-by-female interactions determine the relative fertilization success of competing males (Clark et al. 1999; Miller and Pitnick 2002; Miller and Pitnick 2003; Otronen et al. 1997), it is unclear what outcome to predict from altering the trait of only one sex. Nevertheless, interpretational caution is warranted until more work can be conducted on these and other systems.

We currently lack any understanding of the adaptive significance of female sperm choice (note that we here exclude consideration of choice for genetic compatibility). In the case of *Drosophila*, for example, SR length/morphology is the proximate basis of female sperm choice for sperm length (Miller and Pitnick 2002). An experimental evolution study has demonstrated a significant developmental time cost to females of growing a longer SR (Miller and Pitnick 2003). In the extreme case of *D. bifurca* with its 58 mm long sperm (Pitnick et al. 1995b), females have 82 mm long SRs (Pitnick et al. 1999a). The existence of substantive costs associated with female discrimination are an important consideration, irrespective of the specific forces acting on the evolution of the preference.

The sexually-selected sperm hypothesis (Keller and Reeve 1995; Pizzari and Birkhead 2002) was proposed to explain the evolution of multiple mating by females. According to this model, to the extent that additive genetic variation underlies differential male fertilization success, female propensity for polyandry is favored because it increases the probability of producing sons with superior fertilizing ability. The model was not intended to explain female-generated

selection for any specific sperm attribute, and any of the traditional models for the evolution of female mating preferences: good genes, runaway selection, sensory exploitation, and sexually antagonistic coevolution, may apply to sperm choice (Miller and Pitnick 2002). A recent comparative study of *Drosophila*, however, reveals how the sexually-selected sperm and good genes models might collectively explain the evolution of female sperm choice for long sperm (Schoff et al. 2006). Interestingly, central to this explanation is the negative relationship between sperm size and the number of sperm produced. Even extreme variation in the developmental environments encountered by males has little impact on sperm size (Amitin and Pitnick 2006; Gage and Cook 1994). However, the number of sperm produced by males is highly condition-dependent (e.g., (Gage and Cook 1994). Across eight species of *Drosophila*, nearly all of the interspecific variation in the level of condition-dependence of the number of sperm produced and transferred to females was explained by relative testis mass (which is predominantly associated with sperm length; Pitnick 1996). In other words, when sperm are "cheap," any male can produce and inseminate a great quantity. But when each sperm is "expensive," only high quality males (Hunt et al. 2004; Tompkins et al. 2004) can produce a large quantity. A long-sperm preference may thus be a form of indirect mate choice: by evolving biases in favor of longer sperm, females can turn raffle based sperm competition into a mechanism of discrimination for high quality sires (Schoff et al. 2006; Wiley and Poston 1996).

Tables and Figures

Table 1. Specified proportion (p) of long sperm in mixed distributions, estimated proportion of long sperm by the EM algorithm and 90% confidence intervals for the estimates for the simulation study.

Specified p	Estimated p	90% CI
0.10	0.16	0.06 to 0.25
0.30	0.34	0.21 to 0.42
0.50	0.52	0.37 to 0.68
0.70	0.69	0.59 to 0.80
0.90	0.88	0.77 to 0.97

Table 2. Analysis of covariance of second male sperm precedence (P2) for postcopulatory sexual selection experiment with fully factorial variation in sperm quality (i.e. length) and sperm quantity. Prior progeny = number of progeny produced prior to remating by female; d.f. = degrees of freedom; MS = type III mean square.

Source	d.f.	MS	F	P
Sperm length	1	0.178	11.834	0.0010
Sperm number	1	0.136	9.021	0.0037
Prior progeny	1	0.216	14.340	0.0003
Length * number	1	0.060	4.006	0.0492
Length * progeny	1	0.077	5.094	0.0271
Number * progeny	1	0.015	1.013	0.3175
Length * number * progeny	1	0.066	4.350	0.0406
Error	71	0.015		

Table 3. Treatment-wide number of sperm measured and EM algorithm estimates of the proportion of long sperm in the proximal and distal regions of the SR.

Female	First male	Second male	N sperm measured		Proportion long sperm	
			Proximal	Distal	Proximal	Distal
long	long	short	1075	2625	0.82	0.45
long	short	long	1026	2005	0.94	0.72
short	long	short	319	1931	0.80	0.52
short	short	long	719	2481	0.89	0.78

Table 4. Mean (actual) sperm head lengths, EM algorithm estimates of the proportion of long sperm in the proximal and distal regions of the SR and of the proportion difference between the two regions, from individual female-level analyses. The F-statistic and P-values are from ANOVAs testing the difference in proportion between proximal and distal regions.

Female	1st male	2nd male	N	Mean head length		Prop. (± SD) long sperm		Prop. (± SD) difference	F	P
				Proximal	Distal	Proximal	Distal			
long	long	short	29	9.97	9.62	0.83 ± 0.24	0.60 ± 0.33	0.22 ± 0.24	57.14	< 0.0001
long	short	long	19	10.07	9.88	0.98 ± 0.03	0.90 ± 0.09	0.08 ± 0.09	33.89	< 0.0001
short	long	short	21	10.05	9.68	0.90 ± 0.15	0.67 ± 0.23	0.23 ± 0.21	42.84	< 0.0001
short	short	long	26	10.15	9.94	0.91 ± 0.19	0.90 ± 0.18	0.02 ± 0.15	18.40	< 0.0001

Figure 1.

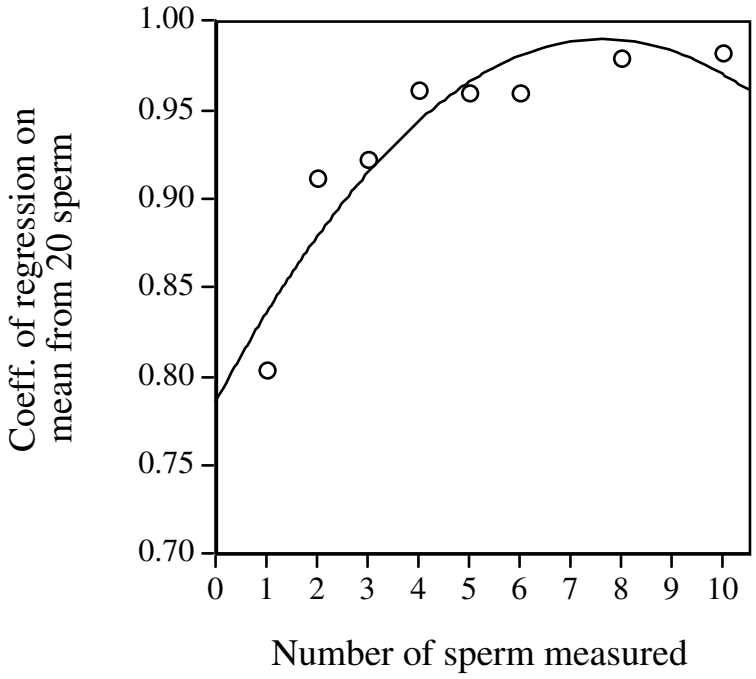

Figure 1. Relationship between the number of sperm assayed and the accuracy of estimation of male sperm length.

Figure 2.

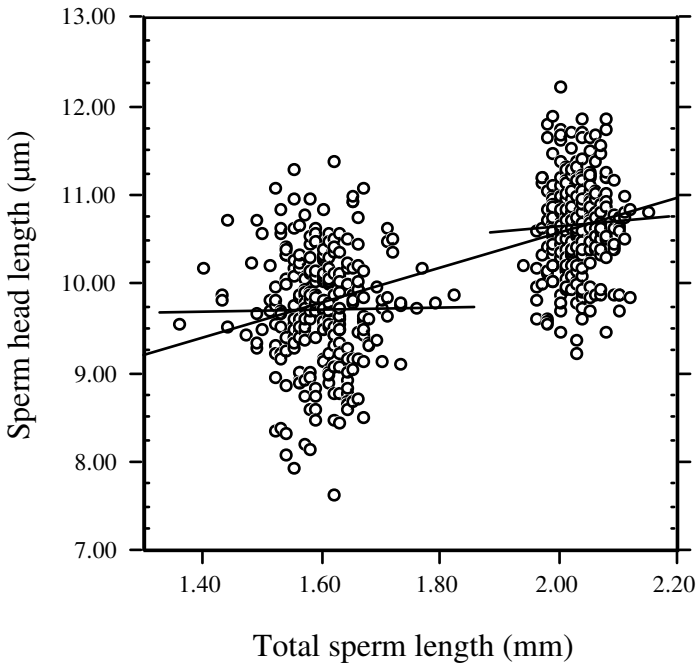

Figure 2. Relationship between sperm head length (µm) and total length of sperm (mm). Best fit lines from least squares regression are shown for analyses of discrete selection lines (solid) and for all sperm from both lines combined (dashed).

Figure 3.

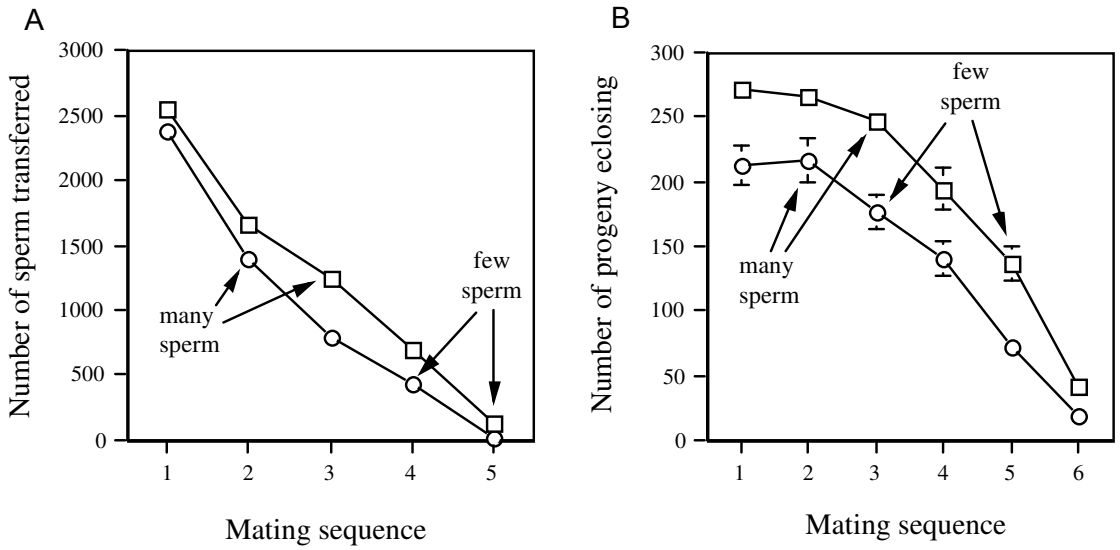

Figure 3. Number of sperm transferred (A) and number of progeny eclosing (B) across a succession of matings by individual males from the short-sperm (circles) and long-sperm (squares) populations. Bars indicate 1 standard error.

Figure 4.

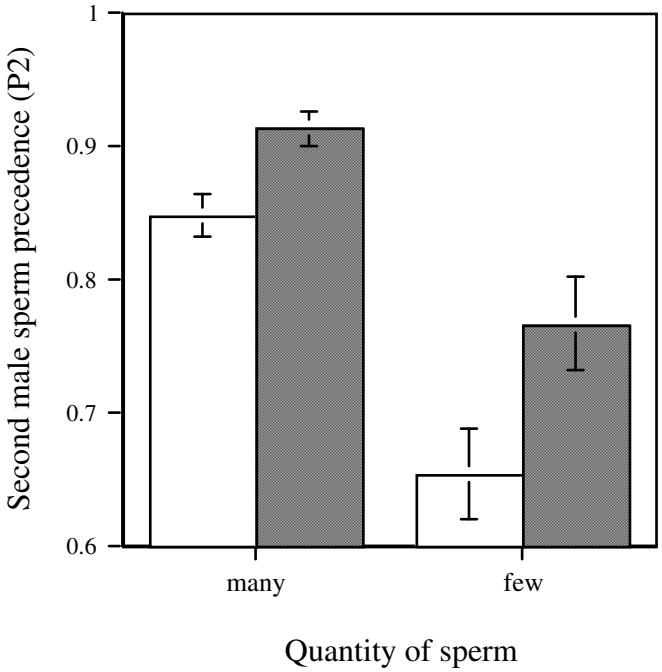

Figure 4. Pattern of sperm precedence with varying sperm quality and quantity. White columns = short-sperm males; gray columns = long-sperm males. Bars indicate 1 standard error. Note: raw P2 scores shown here are for illustrative purposes only; interpretation is based on ANCOVAs of transformed P2 values (see text for details).

Figure 5.

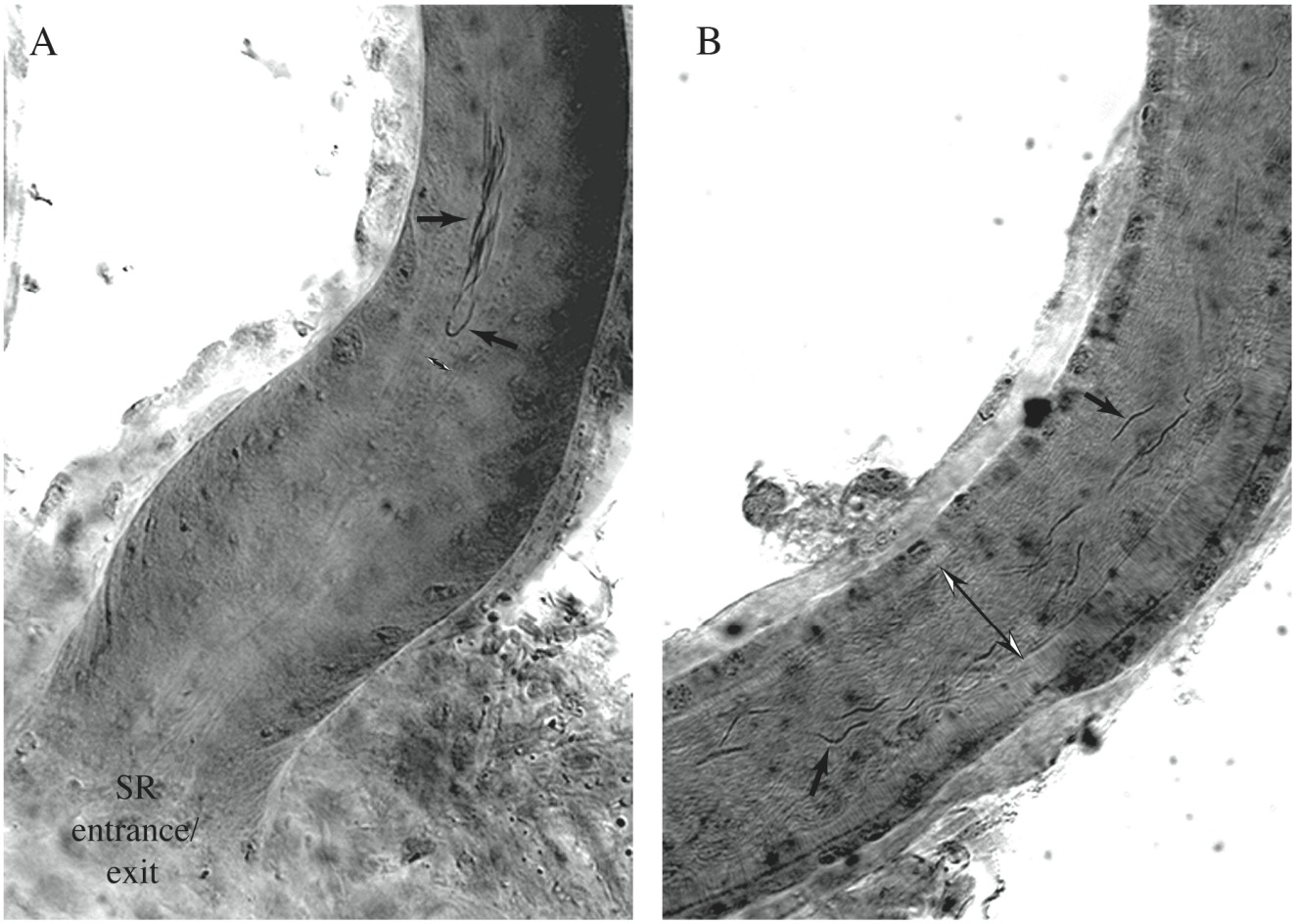

Figure 5. Micrographs showing in vivo organization of aceto-orcein stained sperm heads within the proximate (left) and distal (right) ends of the female's seminal receptacle. Both images were obtained at the same magnification. Arrowheads indicate select sperm; double-headed arrows indicate diameter of SR lumen. Note: because these are "optical slices," only sperm heads positioned within the depth of field are visible.

Figure 6.

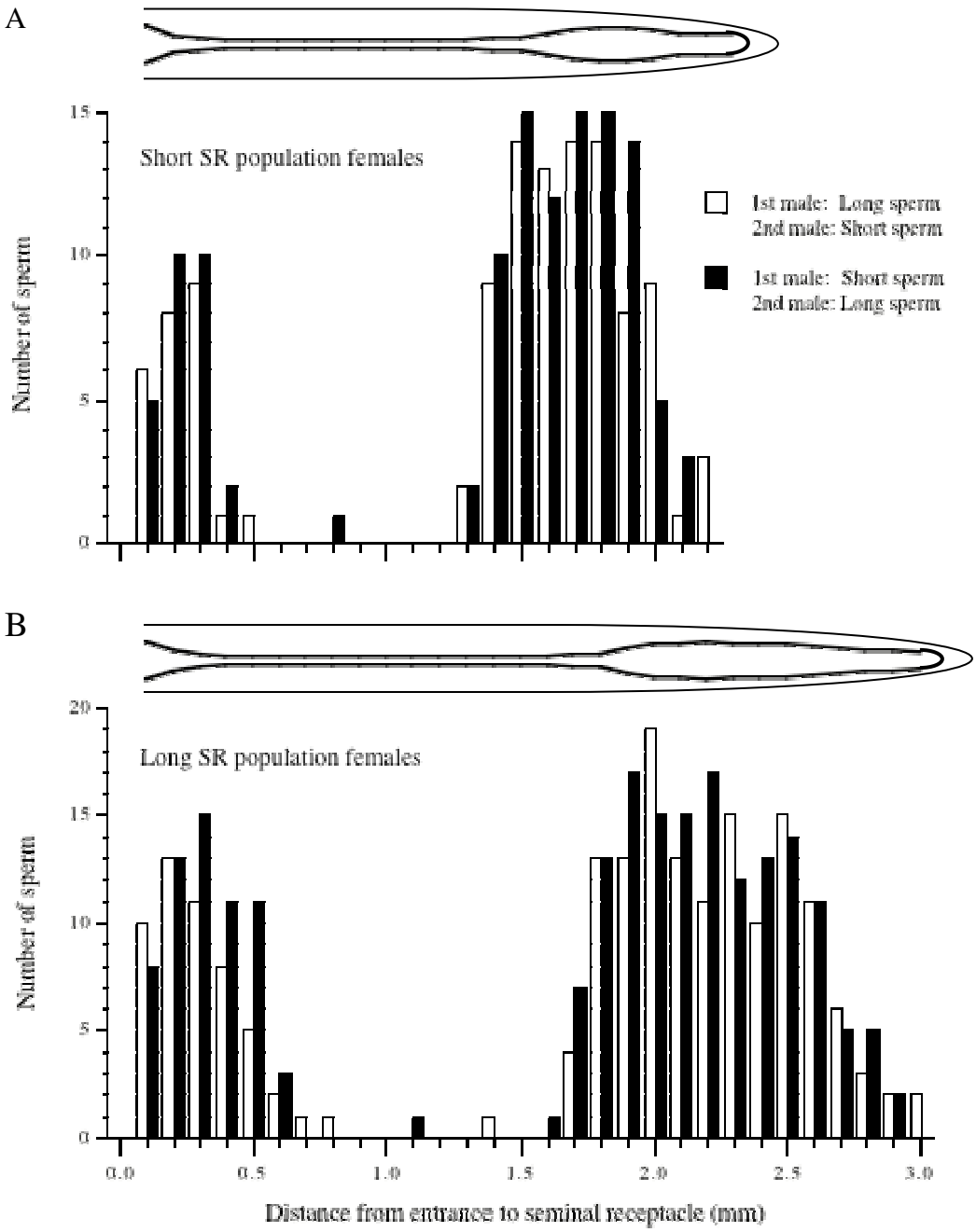
A
Short SR population females
Number of sperm
15
10
5
0
1st male: Long sperm
2nd male: Short sperm
1st male: Short sperm
2nd male: Long sperm
B
Long SR population females
Number of sperm
20
15
10
5
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Distance from entrance to seminal receptacle (mm)

Figure 6. Frequency histograms mapping the distribution of sperm heads throughout the female seminal receptacle for both (A) short-SR population and (B) long-SR population females. All females were doubly mated, either first to a long-sperm male and next to a short-sperm male (white bars) or vice-versa (black bars). See text for details of the mating and dissection procedure. Distance 0.0 indicates the proximate end (i.e., entrance/exit) of the seminal receptacle and the approximate site of egg fertilization. Positioned above each histogram is a schematic illustrating the dimensions of the inner diameter of the lumen for the respective female lines. Each schematic is accurately positioned relative to the x-axis of the respective histograms.

Figure 7.

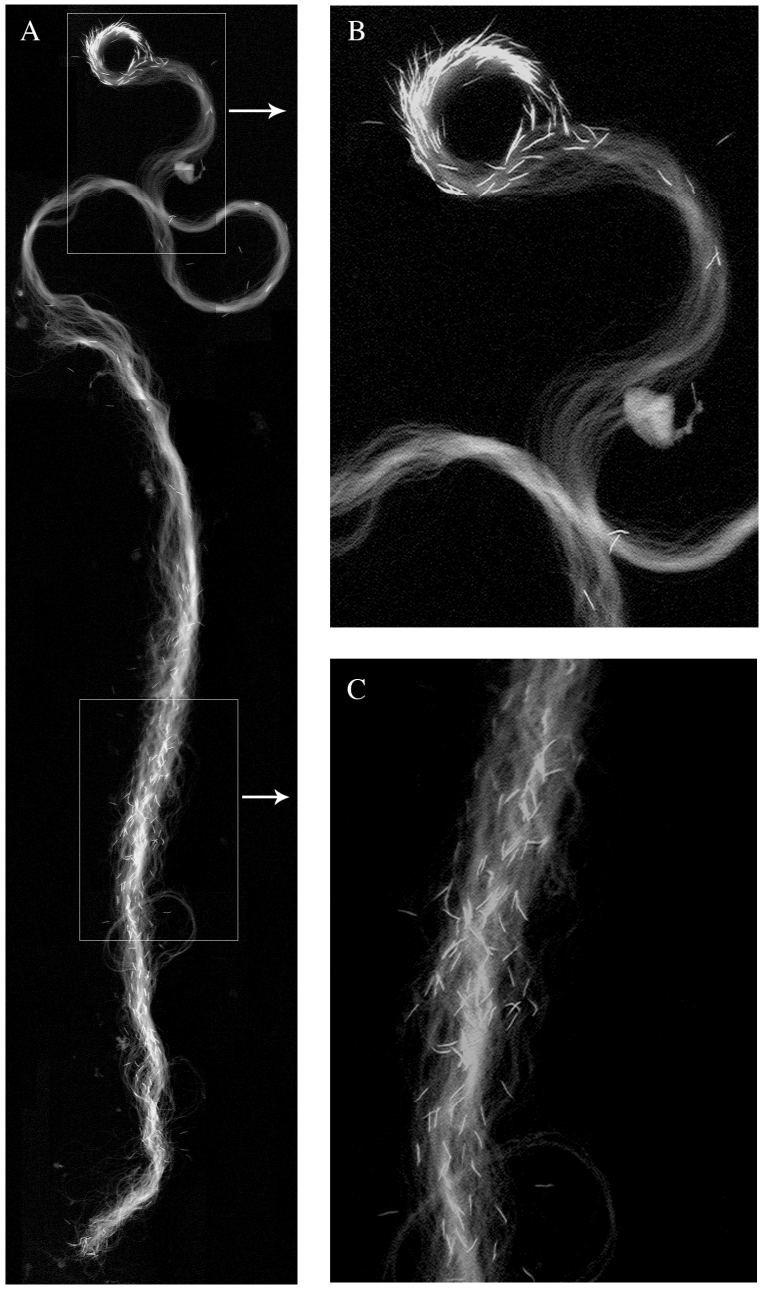
A
B
C

Figure 7. (A) Micrograph of sperm mass removed intact from a female's seminal receptacle without disrupting the relative position of stored sperm. Flourescent sperm heads appear bright white. Mass is oriented with the end occupying the proximal end of the SR at the top of the image. (B) Magnified view of proximal end of sperm mass. Note dense clump of sperm heads at proximal end, followed by region containing the tails of those sperm with only a few additional heads. (C) Magnified view of distal region of sperm mass. Note apparent lack of organization of sperm heads.

Figure 8

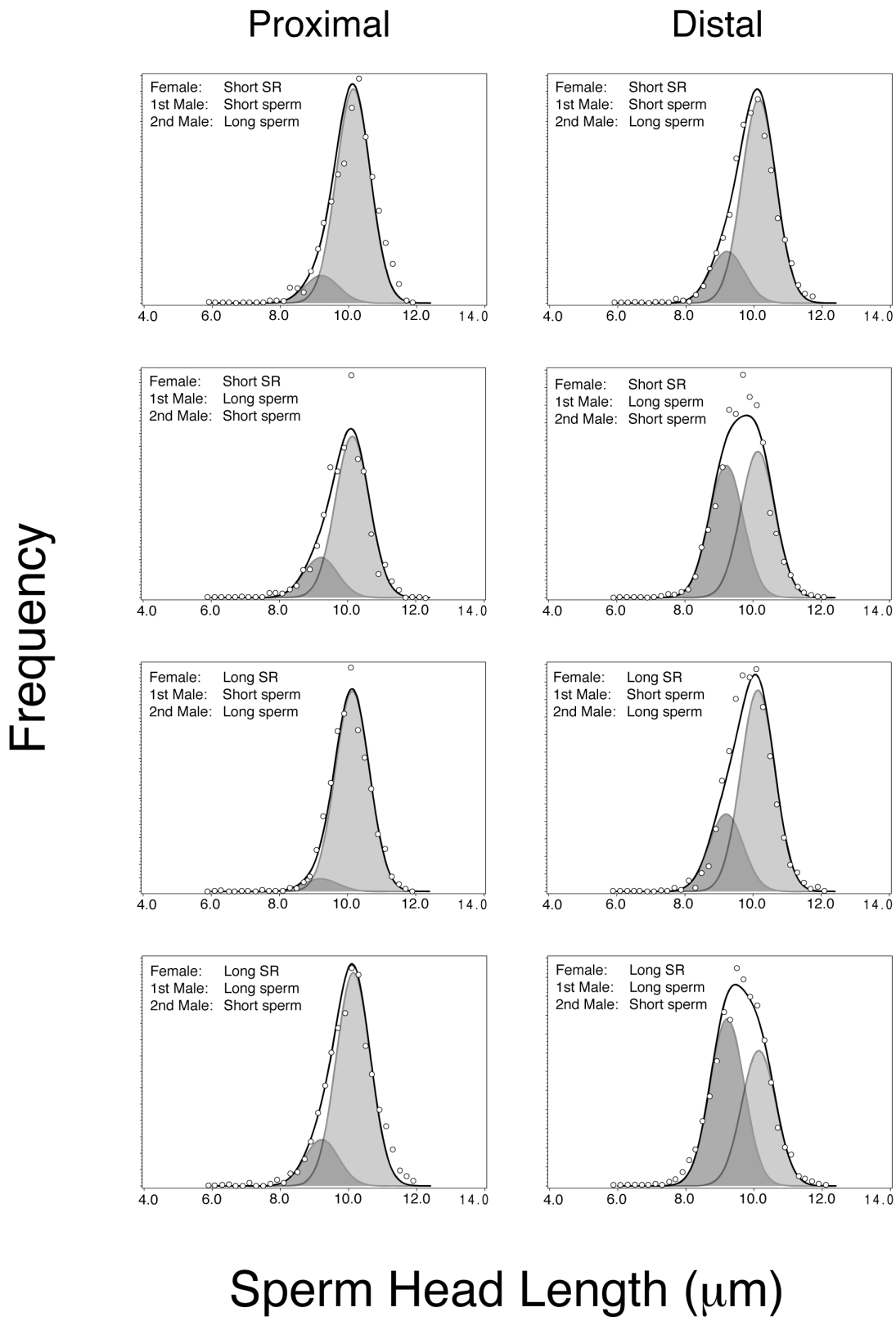
Proximal
Distal
Frequency
Female: Short SR
1st Male: Short sperm
2nd Male: Long sperm
Female: Short SR
1st Male: Long sperm
2nd Male: Short sperm
Female: Long SR
1st Male: Short sperm
2nd Male: Long sperm
Female: Long SR
1st Male: Long sperm
2nd Male: Short sperm
4.0
6.0
8.0
10.0
12.0
14.0
Sperm Head Length (μm)

Figure 8. Decomposition of the distribution for sperm head lengths measured in the proximal (left) and distal (right) regions of the SR for all females from each of the four mating treatments. The empirical distribution of the data is shown by open white circles. Data analyses on each sperm type (measured alone in individual females) showed the distributions of each to be normally distributed with standard deviations of ~0.5. We thus assumed a mixture of two normal distributions each with a variance of 0.25 in the decomposition algorithm. The dark grey distribution represents the sperm from short-sperm line males, the light grey distribution represents the sperm from long-sperm line males and the white distribution represents the sum of the two distributions.

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