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WHAT USE IS AN INFERTILE SPERM? A COMPARATIVE STUDY OF SPERM-HETEROMORPHIC *DROSOPHILA*

Luke Holman,[1] Robert P. Freckleton,[1] and Rhonda R. Snook[1,2]

[1] *Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, United Kingdom*

[2] *E-mail: r.snook@shef.ac.uk*

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Sperm size and number are important determinants of male reproductive success. The genus *Drosophila* exhibits a remarkable diversity of sperm production strategies, including the production of multiple sperm morphs by individual males, a phenomenon called sperm heteromorphism. Sperm-heteromorphic *Drosophila* species in the *obscura* group produce large numbers of infertile "parasperm" in addition to fertile eusperm. Parasperm have been hypothesized to perform a number of roles in place of fertilization, predominantly focused on their potential function in postcopulatory sexual selection. However, the evolutionary significance of parasperm remains unknown. Here we measured several male and female morphological, behavioral, and life-history traits in 13 *obscura* group species to test competing hypotheses of parasperm function using comparative methods. We found that parasperm size was unrelated to female reproductive tract morphology but was negatively related to our two indices of sperm competition, suggesting that postcopulatory sexual selection may indeed have shaped the evolution of parasperm. We also found abundant coevolution between male and female reproductive traits. Some of these relationships have been found in both sperm-monomorphic and sperm-heteromorphic taxa, but others are dissimilar. We discuss the significance of our results to the evolution of reproductive traits and the elusive function of *Drosophila* parasperm.

KEY WORDS: **Drosophila, aesperm, life history, parasperm, reproduction, sperm dimorphism, sperm heteromorphism, sperm morphology, sperm polymorphism.**

Sperm cells possess a remarkable degree of variation in size and shape, and determining the causative factors of this variability has been the subject of much recent research (e.g., Birkhead and Møller 1998; Miller and Pitnick 2002; Gage and Morrow 2003; Snook 2005; Bjork and Pitnick 2006). Sperm morphology is typically uniform within individuals (Gage 1994; Hosken 1997; Ward 1998; Snook 2001), but in some species males produce ejaculates containing distinct sperm morphs, a phenomenon known as sperm heteromorphism. Sperm heteromorphism is well documented in insects (Swallow and Wilkinson 2002; Till-Bottraud et al. 2005) and molluscs (Buckland-Nicks 1998; Oppliger et al. 2003), and has been found in many other taxa (Till-Bottraud et al. 2005). There are usually two distinct sperm morphs, but in some taxa there may be more. In species in which the fertilization competence of the different sperm morphs has been assessed, only one morph participates in fertilization (Till-Bottraud et al. 2005). The fertile sperm types are called "eusperm" and the infertile types are "parasperm" (Healy and Jamieson 1981).

A number of hypotheses have been proposed to explain the persistence of a sterile caste of sperm across taxa (Silberglied et al. 1984; Swallow and Wilkinson 2002; Till-Bottraud et al. 2005; Holman and Snook 2006). For example, parasperm might transfer nutriment to the female, her eggs or the fertile eusperm, or facilitate eusperm transport. However, the hypotheses that have received the most attention focus on the potential role of parasperm in postcopulatory sexual selection (e.g., Gage 1994; Snook 1998a; Cook and Wedell 1999; Presgraves et al. 1999; Morrow and Gage 2000; Oppliger et al. 2003). For example, parasperm could increase the success of eusperm in sperm competition, either by depressing female remating behavior (the "cheap filler" hypothesis) or by

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displacing, blocking, or killing rival eusperm (Silberglied et al. 1984). Parasperm might also improve eusperm survival inside the female reproductive tract (Holman and Snook 2006) thereby improving males' success in numerical sperm competition (Parker 1998), or allow males to gain extra fertilizations through cryptic female choice (Holman and Snook 2006). Despite both comparative (Gage 1994; Presgraves et al. 1999; Morrow and Gage 2000) and intraspecific tests of parasperm function (Snook and Markow 1996; Snook 1998a; Cook and Wedell 1999; Oppliger et al. 2003), there are no sperm-heteromorphic taxa in which the function of parasperm is fully understood (Till-Bottraud et al. 2005; Holman and Snook 2006).

Sperm heteromorphism occurs in the *obscura* group, a monophyletic clade in the genus *Drosophila* that contains 35 described species (Barrio and Ayala 1997). Every species in this group so far examined produces two types of sperm, a long morph and a short morph (Joly and Lachaise 1994; Snook 1997). The sizes of the two morphs are entirely discrete except in *D. subobscura* where partial overlap occurs (Snook 1997). The two sperm types have relatively similar ultrastructure and both types carry a normal haploid complement of chromosomes (Takamori and Kurokawa 1986; Pasini et al. 1996). However, the shorter type is infertile; only long sperm are ever found inside eggs (Snook et al. 1994; Snook and Karr 1998; Snook and Markow 2002), and hybrid males (from a cross of two *D. pseudoobscura* subspecies) that produce only the short morph are infertile (Snook 1998b). The mechanism for fertilization incompetence is unclear, but the head of the parasperm appears to be wider than eusperm (Takamori and Kurokawa 1986; Pasini et al. 1996) so parasperm may be unable to enter the micropyle of the egg (Snook and Karr 1998).

Infertile sperm in this taxon are unlikely to be nonadaptive errors of spermatogenesis (Harcourt 1991) because, (1) production of the two morphs appears to be tightly regulated (Beatty and Burgoyne 1971; Takamori and Kurokawa 1986), (2) there is both intra- and interspecific variation in the proportion of parasperm in an ejaculate (Joly and Lachaise 1994; Snook 1997; Snook and Markow 2001) and (3) infertile sperm number may negatively covary with fertilizing sperm number, a key component of male reproductive success (Parker 1998). Given (1–3), selection would presumably reduce or cease parasperm production if it was not advantageous. Thus, parasperm are likely to be adaptive in the *obscura* group. However, as in other sperm-heteromorphic taxa, the adaptive function(s) of parasperm has remained elusive. Two adaptive hypotheses have been directly tested and these were not supported. Parasperm do not appear to transfer any nutriment to females, as shown by experiments using *D. pseudoobscura* with radioactively tagged ejaculates (Snook and Markow 1996) and probably do not function as cheap filler in postcopulatory sexual selection as the number of sperm in storage does not differ between *D. pseudoobscura* females that are either receptive or refractory to a second mating (Snook 1998a).

Here, we address the conundrum of parasperm function in the *obscura* group interspecifically using the comparative method. Comparative analyses have previously been used to uncover patterns of evolution in sperm-heteromorphic Lepidoptera (Gage 1994; Morrow and Gage 2000) and Diopsidae (stalk-eyed flies; Presgraves et al. 1999). We build upon this approach by using recently developed comparative techniques and by quantifying more traits than previous studies. We measured several morphological, behavioral, and life-history characters, namely the length and relative abundance of the two sperm morphs, female sperm storage organ dimensions, male reproductive tract mass, female remating rate, age at reproductive maturity, copulation duration, and body size, that allow us to test hypotheses on the functional significance of sperm heteromorphism.

Using these data, we tested for associations between traits that are predicted by competing hypotheses of parasperm function. We estimated the strength of sperm competition by quantifying the relative mass of the male reproductive tract (see Parker et al. 1997) and female remating rate (e.g., Gage 1994). If parasperm function in postcopulatory sexual selection, then we should see an association between these sperm competition measures and parasperm traits such as length and proportion of parasperm. Specifically, if parasperm function as "displacers" of rival eusperm or enhance male success in cryptic female choice, then males may produce larger/more parasperm when postcopulatory sexual selection is strong, assuming a greater quantity of parasperm results in increased functionality (Holman and Snook 2006). Under the hypotheses that parasperm function as either "cheap filler" or blockers, we predict parasperm size and/or abundance to have coevolved with female sperm storage organ size across species (Presgraves et al. 1999); parasperm traits may also be related to female remating rate if parasperm are cheap filler. If parasperm protect brother eusperm from spermicide, then males should produce more parasperm as eusperm become more vulnerable to spermicide. We evaluate these predictions and also compare trends in the evolution of key reproductive traits among different sperm-heteromorphic taxa, and between sperm-monomorphic and sperm-heteromorphic taxa.

Methods

FLY CULTURES

We obtained the following species from the Tucson *Drosophila* Stock Center: *D. affinis* (stock no. 141.2), *D. ambigua* (14011–0091.1), *D. guanche* (14011–0095), and *D. miranda* (14011–0101.7). The other stocks were obtained from a variety of sources; *D. athabasca* and *D. azteca* were collected in the USA in 2000, *D. imaii* was collected in Sapporo, Japan in 1972, *D. obscura* and *D. tristis* were collected in Tübingen, Germany in 1981,

D. persimilis was collected in Mather, California in 1998, *D. pseudoobscura* was collected in Tempe, Arizona in 1998, *D. subobscura* was collected from Valdivia, Spain in 2000, and the source of the *D. tolteca* stock is unknown. All fly cultures were kept in 24 × 80 mm polypropylene vials in a 12:12 light:dark photocycle with an approximately equal sex ratio and we endeavored to minimize inbreeding. All species were provided with live yeast and standard cornmeal, agar, and molasses food medium, except for *D. athabasca*, *D. azteca*, and *D. tolteca*, which were kept on yeasted potato-based instant medium. The flies were incubated at 18°C, with the exception of *D. affinis*, *D. persimilis*, *D. pseudoobscura*, and *D. tolteca*, which were kept at 22°C. We attempted to investigate additional species, but some stocks were not available or could not be successfully cultured.

AGE AT REPRODUCTIVE MATURITY

Newly eclosed, virgin flies were collected from the stocks under mild CO_2 anesthesia and placed in yeasted single-sex vials to mature. To determine how many days after eclosion males and females became reproductively mature, we conducted a series of mating trials. To find female age at reproductive maturity, we placed one female of known age with two males, estimated to be mature, in each of 25–30 test vials and observed the flies for 2 h. When copulations occurred, the nonmating male was removed by aspiration and the duration of the copulation was recorded to the nearest minute. Female flies were judged to be reproductively mature if 80% or more mated during the 2h observation period (e.g., Pitnick et al. 1995; Snook and Markow 2001). We sought the youngest females that displayed a $\geq 80\%$ mating rate; for example, if we observed that the incidence of mating was 40% in a sample of two-day-old females and 90% in three-day-old females, we would record the female age at reproductive maturity as three days in that species. We repeated this procedure with groups of two females and one male to determine male age at reproductive maturity. If 80% or more of the males mated, we dissected their mates and checked for the presence of mobile sperm in the uterus. Male flies were subsequently judged to be reproductively mature if 80% of them mated *and* transferred mobile sperm. After determining male age at reproductive maturity, we verified that no immature males were used in the previous assays of female age at reproductive maturity. If immature males had been used, we repeated the female age at reproductive maturity trials using mature males. In this iterative way, an accurate measure of both male and female age at reproductive maturity was determined. All flies used in subsequent assays were 1 day older than the age at reproductive maturity unless stated otherwise.

MALE REPRODUCTIVE TRACT AND BODY MASS

Male flies were dissected in distilled water on a piece of preweighed foil. The testes, seminal vesicles, accessory glands, and the ejaculatory bulb were removed and transferred together to another piece of foil. The foils holding the body and reproductive system of the fly were then placed in a drying oven at 60°C for 18 ± 1 h and weighed on a Mettler Toledo UMX2 balance to the nearest micrograms (Pitnick 1996). Sample size is 25 males per species. We measured the both testes and accessory glands to quantify male investment in both sperm and nonsperm components of the ejaculate. Previous studies simply examine testes size relative to body size, however, recent research in *Drosophila* (Bangham et al. 2002) and diopsid stalk-eyed flies (Rogers et al. 2005a, b) has indicated that accessory gland size positively responds to sperm competition, as does testes size. We therefore included both in our estimate of the strength of sperm competition.

FEMALE MORPHOLOGICAL MEASUREMENTS

Three measurements of the female reproductive tract were made in each species: the length of the ventral receptacle (VR; also known as the seminal receptacle), the area of the spermathecae, and the length of the spermathecal ducts, using a method similar to that of Miller and Pitnick (2003). The reproductive system (minus ovaries) was removed by pulling on the ovipositor then transferred to a 20 μl drop of phosphate-buffered saline (PBS) on a slide. The tissue surrounding the sperm storage organs, that is, the uterus and the common oviduct, was then excised. After applying a coverslip, a randomly chosen spermatheca was photographed at 400× magnification. Next, the PBS under the coverslip was drawn off with tissue paper to flatten the reproductive tract. The VR and one randomly chosen spermathecal duct were then photographed at 400×. To assess the repeatability of this method, we measured the VRs of 10 *D. affinis* females twice (repeatability = 0.85, $F_{9,10} = 3.02$, $P < 0.001$; calculated as in Lessells and Boag 1987). This was accomplished by drawing off the PBS, taking the first picture, removing the coverslip, and adding another 20 μL of PBS, then drawing off the PBS again and taking a second picture. We also quantified female thorax length. We photographed the dorsal surface of anaesthetized females at 40× magnification then measured the thorax using Scion Image (repeatability = 0.97; $F_{9,10} = 65$, $P < 0.0001$). The sample size was 30 females per species.

FEMALE REMATING RATE

Thirty-five virgin females were each placed in yeasted food vials with two virgin males. When copulations occurred, the nonmating male was removed and the copulation duration was recorded. On every subsequent morning, we introduced two virgin males (aged between one and four days older than reproductive maturity) to each vial and observed the flies for 2 h; remating females were removed from the trial. The trial finished when 50% or more of the females had remated; the number of days between the initial matings and the end of the experiment was used as a measure

of remating interval (Snook and Markow 2001). We calculated "remating rate" by taking the reciprocal of remating interval for use in the analysis because *D. subobscura* is monogamous (see Table 1); this allowed us to record the remating rate of *D. subobscura* as zero (i.e., $1/\infty$) and place it on a scale with the other species. The food vials in which the females were housed were checked for subsequent progeny production, allowing us to exclude females that produced no progeny from their first mating (an indication that the first mating was probably incomplete). We used data from Snook and Markow (2001) to obtain values for remating rate for *D. persimilis* and *D. pseudoobscura*. We also obtained values for species mean copulation duration by compiling data from the remating assays and the age at reproductive maturity trials. Only copulations in which both flies were virgin and reproductively mature were included in the analysis.

SPERM MORPHOLOGY AND DEMOGRAPHY

Where available, we used previously published data on sperm lengths and proportion of parasperm produced. We prioritized publications that measured individual sperm as opposed to sperm bundles. Table 1 gives the origins of the previously published sperm trait data; for the remaining species, sperm head length, tail length, and number of each type transferred to the female were measured as follows.

Sperm was obtained from the uterus of a female 0.5–1.5 h after mating and diluted in 100 μL PBS. The sperm mass was gently teased apart with dissecting pins and mixed by drawing it in and out of a pipette. We placed 2.5 μL of the sperm solution on one slide (for sperm counts) and put the remainder on a second slide with 30 μL of glycerine, which facilitates the staining of the sperm tail for sperm length measurements. The slides were then processed and stained with DAPI (4′-6-Diamidino-2-phenylindole) for sperm counts (Snook et al. 1994; Snook 1997). We counted sperm from 10 males per species at 400× magnification under UV illumination (counts were repeatable; eusperm: $r = 0.84$, parasperm: $r = 0.82$, both $P < 0.001$). To measure eusperm and parasperm length, we photographed 10 eusperm and 10 parasperm from each of 10 males at 400× and measured them using Image Pro Plus software.

PHYLOGENETIC CONTROL AND STATISTICAL ANALYSIS

We used a generalized least squares (GLS) approach to control for phylogenetic dependence (Felsenstein 1985; Harvey and

Table 1. The table gives mean values ± standard error where applicable. Samples sizes are $n = 30$ for the female reproductive tract measurements, $n = 25$ for male reproductive tract mass, and $n = 10$ males for the sperm trait data. VR = ventral receptacle and ARM = age at reproductive maturity. Data with superscripts were collated from previous publications: A = Snook (1997), B = Joly and Lachaise (1994), C = Snook and Markow (2001) and D = Snook (1995). The table also shows the phylogeny of the group (O'Grady 1999) and the maximum likelihood lambda values of each trait.

	Sperm length (μm)						% Parasperm produced	Male Reproductive Tract Mass (μg)	♀ Reproductive tract dimensions (μm and μm²)			Remating interval (days)	Copulation duration (min)	ARM (days)	
	Eusperm			Parasperm											
	Head	Tail	Total	Head	Tail	Total			VR length	Spermathecal area	Spermathecal duct length			♂	♀
affinis	91^{A}	414^{A}	506^{A}	20^{A}	110^{A}	130^{A}	72^{C}	11 ± 0.8	1368 ± 22	2770 ± 67	304 ± 6.0	5 (n = 30)	1.6 ± 0.1 (n = 66)	4	5
athabasca	449^{A}	1088^{A}	1527^{A}	16^{A}	102^{A}	118^{A}	94 ± 3	13 ± 0.8	3564 ± 60	4150 ± 48	256 ± 5.7	3 (n = 29)	9.6 ± 0.3 (n = 97)	8	6
azteca	351^{A}	1097^{A}	1433^{A}	15^{A}	159^{A}	174^{A}	88^{B}	17 ± 0.7	3654 ± 58	3742 ± 60	272 ± 6.5	3 (n = 30)	7.6 ± 0.2 (n = 87)	4	5
tolteca	56 ± 0.77	247 ± 3.4	303	15 ± 0.43	83 ± 2.5	98	69 ± 3	15 ± 1.1	517 ± 9.2	3920 ± 77	229 ± 4.6	4 (n = 28)	7.8 ± 0.2 (n = 130)	4	4
persimilis	70^{A}	254^{A}	324^{A}	13^{A}	64^{A}	77^{A}	50^{C}	26 ± 1.0	586 ± 8.3	4591 ± 50	253 ± 4.5	3^{C} (n = 30)	8.5 ± 0.6 (n = 98)	4	4
pseudoobscura	70^{A}	293^{A}	362^{A}	14^{A}	78^{A}	92^{A}	48^{C}	22 ± 0.8	522 ± 7.7	5429 ± 79	162 ± 2.7	3^{C} (n = 30)	5.4 ± 0.1 (n = 98)	5	6
miranda	61^{A}	248^{A}	309^{A}	13^{A}	74^{A}	87^{A}	56 ± 3	41 ± 1.8	564 ± 8.3	4913 ± 39	236 ± 3.1	7 (n = 30)	7.6 ± 0.3 (n = 80)	4	5
ambigua	59^{A}	253^{A}	313^{A}	16^{A}	74^{A}	94^{A}	73 ± 2	24 ± 0.9	548 ± 7.5	2931 ± 49	228 ± 5.0	8 (n = 29)	0.18 ± 0.01 (n = 22)	6	9
obscura	51^{A}	180^{A}	230^{A}	23^{A}	73^{A}	96^{A}	34^{B}	28 ± 1.6	498 ± 6.8	4995 ± 90	264 ± 4.9	6 (n = 30)	8.6 ± 0.4 (n = 60)	2	4
tristis			235^{B}			112^{B}	67^{B}	16 ± 0.9	532 ± 13	2114 ± 32	251 ± 6.5	7 (n = 28)	6.3 ± 0.2 (n = 86)	2	3
imaii	65 ± 1.6	143 ± 3.96	208	19 ± 0.70	50 ± 2.03	69	42 ± 4	20 ± 1.3	573 ± 12	2299 ± 64	243 ± 4.9	11 (n = 29)	9.9 ± 0.5 (n = 28)	6	7
subobscura	72^{A}	334^{A}	408^{A}	26^{A}	200^{A}	227^{A}	66^{D}	10 ± 0.4	546 ± 10	2767 ± 57	244 ± 4.5	n/a (n = 25)	4.7 ± 0.2 (n = 134)	3	6
guanche			273^{B}			131^{B}	50^{B}	15 ± 0.9	483 ± 7.5	2626 ± 44	244 ± 9.1	9 (n = 30)	8.5 ± 0.2 (n = 78)	6	6
Lambda			0.65			1.00	0.51	1.00	0.85	0.28	0.00	0.94	0.00	1.00	0.68

Pagel 1991), measured by the parameter lambda (λ), in our log-transformed dataset (Pagel 1999; Freckleton et al. 2002). Lambda is the transformation, estimated using maximum likelihood, that makes the trait data fit the Brownian motion evolutionary model. For traits evolving under Brownian motion, lamda is predicted to be 1; for traits that have evolved entirely independently of phylogeny lamda is 0. This approach allows a variable degree of phylogenetic correction, making it preferable to other methods because different traits may not have the same level of phylogenetic dependence. The method for estimating lambda, along with worked examples, can be found in Freckleton et al., (2002). Lambda values and GLS models were computed using code written by R.P. Freckleton implemented in R 2.3.1 software. The phylogenetic topology of O'Grady (1999) was used, with branch lengths set equal (i.e., we assumed speciational evolution).

We found that many traits of interest were correlated with body size (see Results) so we controlled for body size in all models by including it as a factor. When examining the relationship between two male traits, body size was measured by male dry body mass (minus the reproductive organs; body mass and male reproductive tract mass are therefore statistically independent); in relationships between two female traits we used female thorax length. When analyzing the relationship between male and female traits, we controlled for male body mass alone to preserve power and avoid collinearity (male body mass and female thorax length were highly correlated; $r = 0.91$). To reduce the chance of committing type II errors, we did not use Bonferroni correction or related techniques (Nakagawa 2004). Instead, we calculated the effect size correlation, r, from t values (Cohen 1977; Colgrave and Ruxton 2003; Immler and Birkhead 2007; Stephens et al. 2007); effect sizes of ≥ 0.5 are considered large for most purposes (Cohen 1977). We also present confidence limits (CLs) for effect size to illustrate the range of statistically supported effect sizes.

Results

The means and standard errors of the measured traits are shown in Table 1. Our stock of *D. subobscura* was found to be monogamous, as reported for other cultures (Maynard Smith 1956; Markow and O'Grady 2005); none of the 25 females that survived over the 14-day observation period remated. Also, the copulation duration of *D. obscura* was remarkably brief compared to other *Drosophila*, lasting an average of 11 sec. We verified that sperm transfer and subsequent offspring production did occur following these short matings. Lambda values were high for most traits (Table 1).

We detected coevolution among many traits in our dataset (summarized in Table 2), with the exception of spermathecal duct length, copulation duration, and both male and female age at reproductive maturity, which were unrelated to any of the other traits. We will therefore not present further data on these traits.

Eusperm length was very strongly correlated with the length of the female VR (Fig. 1A; Table 2), but unrelated to spermathecal area. Eusperm length also decreased with male body mass (Fig. 2A), but did not covary with either of our measures of the strength of sperm competition (female remating rate and male reproductive tract mass, which were positively related to each other; Table 2). Eusperm length and parasperm length were not significantly related (Table 2).

Parasperm length declined as female remating rate increased (Fig. 3A; Table 2), although this relationship was driven by *D. subobscura*, which has the longest parasperm and does not remate. There was also a borderline significant ($P = 0.056$) trend toward a negative relationship between parasperm length and male reproductive tract mass (Fig. 3B; Table 2). The observed effect size for this model was large ($r = 0.56$) and the effect size CLs do not span zero; taken together, these results suggest that male reproductive tract mass and parasperm length probably are significantly negatively related. None of the measures of female reproductive tract morphology correlated with parasperm length (Table 2). Like eusperm length, parasperm length varied inversely with body mass (Fig. 2B; Table 2).

Parasperm proportion varied with eusperm length such that males with long eusperm had a higher proportion of their ejaculate composed of parasperm (Fig. 4; Table 2). Also, larger species produced a lower proportion of parasperm (Fig. 2C; Table 2). Parasperm proportion was not related to parasperm length, the strength of postcopulatory sexual selection, or the dimensions of the female reproductive tract (Table 2).

Male reproductive tract mass and female remating rate were positively correlated, that is, species with heavy male reproductive systems had females that remated quickly (Table 2). Male reproductive tract mass was also positively related to male body mass (Fig. 2D) and spermathecal area (Fig. 1B), but was unrelated to VR length (Table 2).

All female reproductive tract measurements were unrelated to female thorax length (Table 2). VR length and spermathecal area were also unrelated to each other (Table 2). Spermathecal area was positively correlated with female remating rate, but VR length was not related (Table 2).

Discussion

THE EVOLUTION OF SPERM HETEROMORPHISM AND THE FUNCTIONAL SIGNIFICANCE OF PARASPERM

Eusperm size in the *obscura* group is considerably more variable than parasperm size; eusperm length varies by greater than

Table 2. Phylogenetically controlled multiple regression models quantifying relationships among reproductive traits in *obscura* species. All models have male body mass as an additional predictor (statistics for body mass not shown), except where male body mass is the predictor of interest, or in models examining relationships between two female traits (in which female thorax length was used instead). There are 11 degrees of freedom for all tests, except tests in which body size is the only predictor (df = 12). The effect size *r* and its confidence limits are presented; *P* values and effect size CLs that indicate statistical significance are shown in bold.

Dependent variable	Predictor	Adjusted R^2	Slope	*t*	*P*	Effect size (*r*)	Effect size CLs
Eusperm length	Parasperm length	0.40	0.64	1.43	0.19	0.41	−0.20 to 0.74
	Male reproductive tract	0.24	0.26	0.30	0.77	0.09	−0.45 to 0.56
	Female remating rate	0.25	0.014	0.08	0.94	0.03	−0.51 to 0.54
	VR length	0.87	0.75	6.26	**0.0002**	0.89	**0.67 to 0.95**
	Spermathecal area	0.25	0.29	0.44	0.68	0.13	−0.42 to 0.59
	Male body mass	0.32	−1.31	−2.43	**0.038**	−0.59	**−0.82 to −0.06**
Parasperm length	Male reproductive tract	0.35	−0.63	−2.24	0.056	−0.56	**−0.80 to −0.009**
	Female remating rate	0.58	−0.26	−3.54	**0.008**	−0.75	**−0.88 to −0.30**
	VR length	−0.04	0.15	0.85	0.42	0.26	−0.34 to 0.67
	Spermathecal area	0.00	−0.29	−0.70	0.50	−0.22	−0.48 to 0.57
	Male body mass	0.44	−0.60	−2.84	**0.021**	0.65	**−5.08 to −0.51**
% Parasperm produced	Eusperm length	0.58	0.31	2.52	**0.036**	0.62	**0.07 to 0.83**
	Parasperm length	0.42	0.61	1.46	0.19	0.40	−0.18 to 0.72
	Male reproductive tract	0.26	−0.09	−0.41	0.69	−0.23	−0.60 to 0.44
	Female remating rate	0.26	−0.03	−0.39	0.71	−0.12	−0.60 to 0.45
	VR length	0.46	0.20	1.79	0.11	0.49	−0.11 to 0.77
	Spermathecal area	0.25	−0.09	−0.28	0.79	0.09	−0.58 to 0.47
	Male body mass	0.33	−0.61	−2.43	**0.038**	−0.59	**−0.82 to −0.06**
Male reproductive tract mass	Female remating rate	0.76	0.28	4.23	**0.003**	0.80	**0.43 to 0.91**
	Ventral receptacle	0.60	−0.05	−0.34	0.73	−0.10	−0.44 to 0.67
	Spermathecal area	0.82	0.58	3.11	**0.017**	0.68	**0.21 to 0.85**
	Male body mass	0.64	1.02	4.14	**0.003**	0.78	**1.49 to 6.69**
Ventral receptacle	Female remating rate	0.14	0.06	0.29	0.78	0.09	−0.45 to 0.56
	Spermathecal area	0.13	−0.05	−0.07	0.94	−0.02	−0.50 to 0.52
	Female thorax length	0.23	−5.64	−1.99	0.08	−0.50	−0.05 to 0.76
Spermathecal area	Female remating rate	0.26	0.19	2.33	**0.048**	0.57	**0.03 to 0.80**
	Female thorax length	−0.11	0.24	0.17	0.87	0.05	−0.40 to 0.52

sevenfold across our sample whereas parasperm length varies threefold, and there was no correlation between the lengths of the two morphs. These results indicate that eusperm and parasperm size have evolved independently of one another and suggest that different selective pressures have shaped the evolution of the two morphs (Snook 1997), as expected if parasperm are adapted to a novel function.

Parasperm length was negatively correlated with both our measures of sperm competition (remating rate and male reproductive tract mass), indicating that if parasperm function in sperm competition, then smaller parasperm are better. Males producing small parasperm could potentially reallocate resources to augment other ejaculatory traits, such as either eusperm or parasperm number, or eusperm length. However, parasperm length is unrelated to either the proportion of parasperm or eusperm length, so we found no evidence that reduced parasperm size is associated with greater investment in these traits.

Interspecific evidence of sperm competition selecting for reduced sperm size is rare; in 13 comparative studies of different sperm-monomorphic vertebrates and invertebrates, seven showed a positive relationship between sperm size and sperm competition, five found no relationship and just one detected a negative relationship (reviewed in Snook 2005). In two similar studies of sperm-heteromorphic Lepidoptera, eusperm length increased with sperm competition strength but parasperm size showed no relationship (Gage 1994; Morrow and Gage 2000). Our finding that eusperm length is unaffected by the strength of sperm competition whereas parasperm length is negatively related is therefore surprising for two reasons. First, we found selection for smaller sperm under increasing sperm competition, in contrast to the majority of

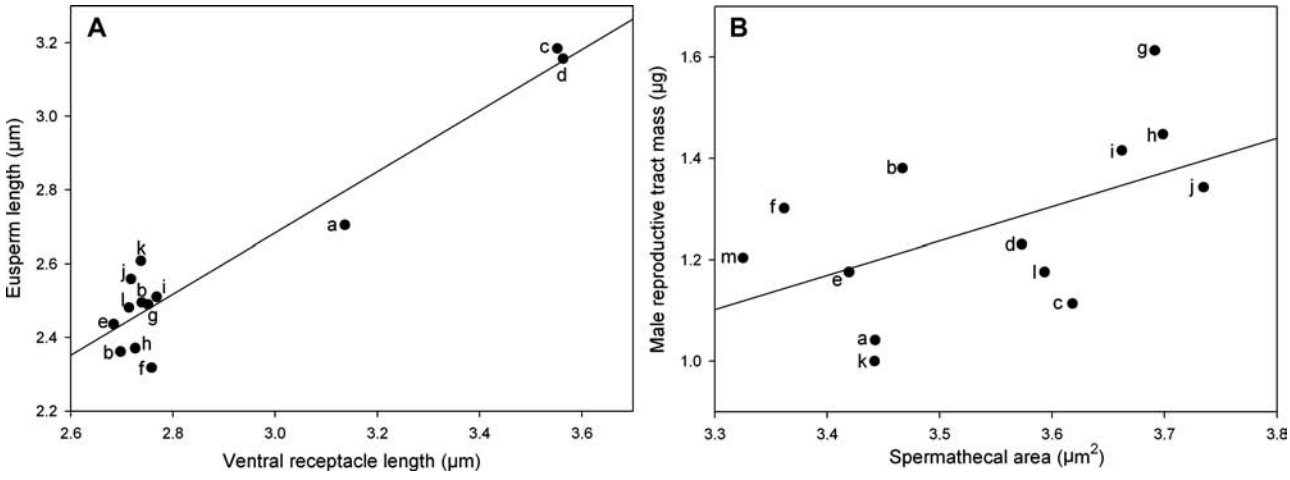

Figure 1. Coevolution was found between female sperm storage organs and male reproductive traits: (A) ventral receptacle and eusperm length; (B) spermathecal area and male reproductive tract mass. The line is from a GLS regression of the log-transformed data. The letters denote species as follows; a, *affinis*; b, *ambigua*; c, *athabasca*; d, *azteca*; e, *guanche*; f, *imaii*; g, *miranda*; h, *obscura*; i, *persimilis*; j, *pseudoobscura*; k, *subobscura*; l, *tolteca*; m, *tristis*.

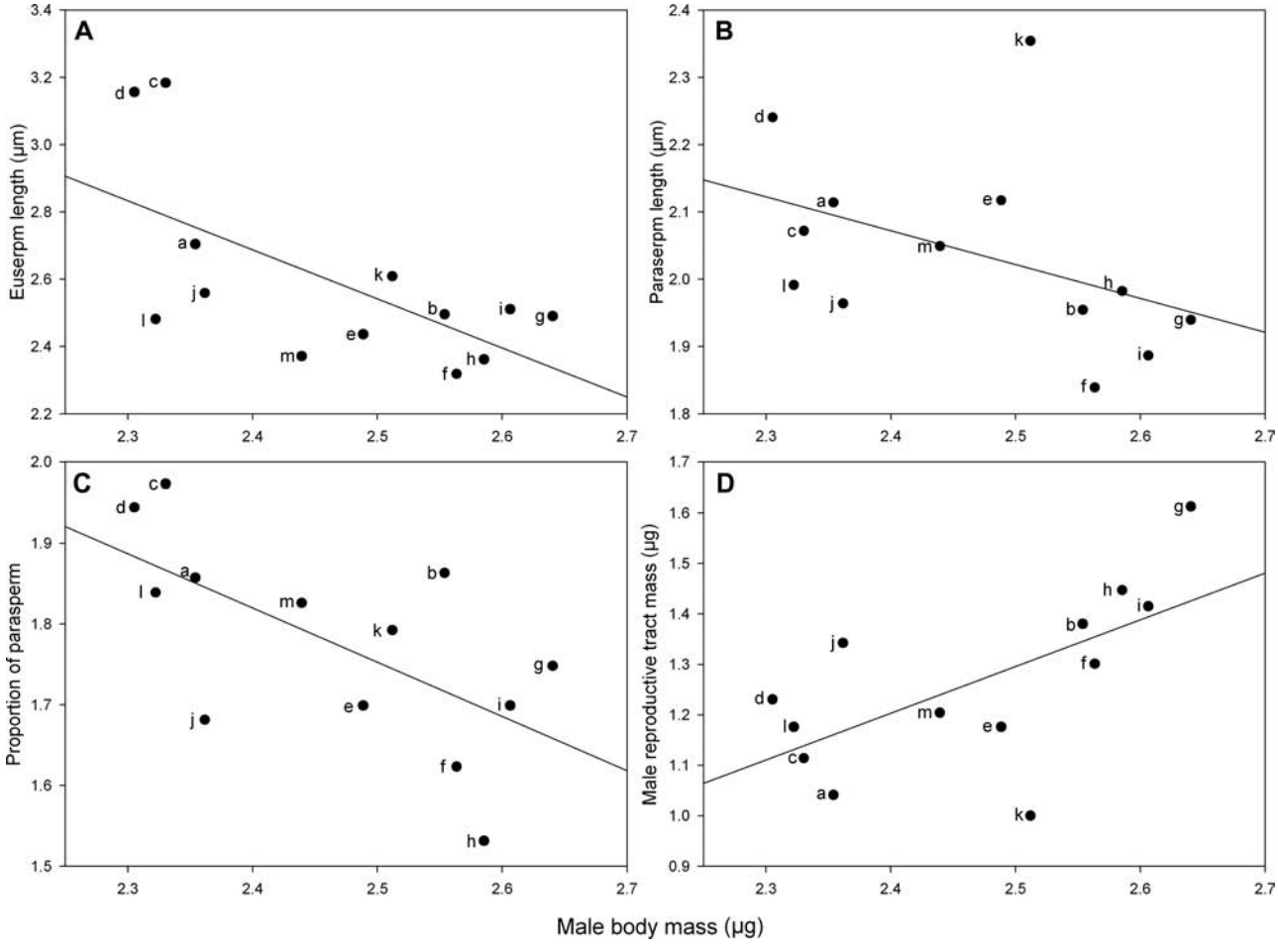

Figure 2. Male body mass is negatively related to the length of both sperm morphs and to parasperm proportion, but is positively correlated with male reproductive tract mass: (A) eusperm length, (B) parasperm length, (C) parasperm proportion and (D) male reproductive tract mass. The lines are from GLS regressions of the log-transformed data. See Figure 1 legend for species codes.

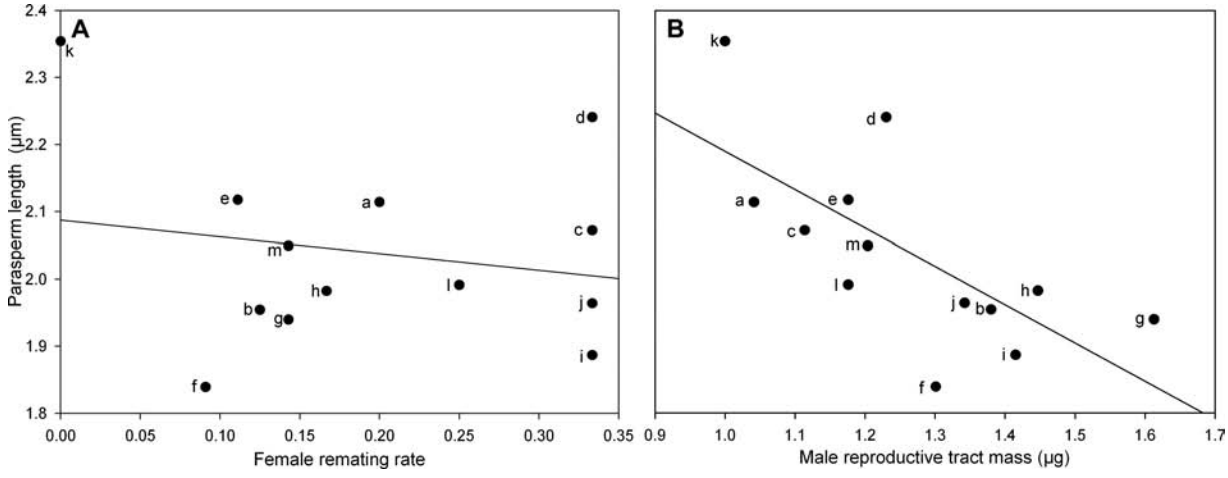

Figure 3. **Parasperm length varies negatively with both measures of sperm competition: (A) female remating rate and (B) male reproductive tract mass. The lines are from GLS regressions of the log-transformed data. See Figure 1 legend for species codes.**

other studies. Second, the evolutionary response of heteromorphic sperm size to sperm competition differs between the *obscura* group and Lepidopterans, suggesting a difference in parasperm function between these taxa.

In sperm-heteromorphic stalk-eyed flies, both eusperm and parasperm lengths are associated with the size of sperm storage organs (Presgraves et al. 1999). The latter relationship was suggested as evidence that parasperm function as cheap filler, filling the female sperm stores to delay her remating (Presgraves et al. 1999). We did not find such a relationship (see also Morrow and Gage 2000), suggesting that parasperm are not cheap filler in *Drosophila*. The cheap filler hypothesis also predicts: (1) negative associations between either (a) parasperm size and/or (b) abundance and female remating rate, and (2) a positive relationship between parasperm abundance and female reproductive tract dimensions. Although we did find a negative relationship between parasperm size and female remating rate, the other predicted relationships were not observed. Therefore, overall, our results suggest that parasperm do not function as cheap filler in the *obscura* group. These results support the conclusion of an intraspecific test of the cheap filler hypothesis in *D. pseudoobscura* (Snook 1998a). The idea that parasperm are "blockers" that impede entry by rival eusperm to the site of fertilization (Silberglied et al. 1984) would similarly predict coevolution between parasperm size/number and female reproductive tract dimensions, and is therefore not supported by this study (see also Snook 1998a).

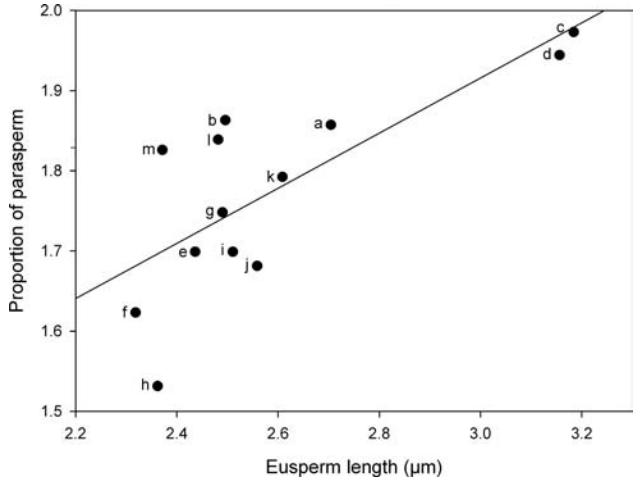

Figure 4. **The proportion of parasperm varies with eusperm length. The ejaculates of species with long eusperm contain a higher proportion of parasperm. The line is from a GLS regression of the log-transformed data. See Figure 1 legend for species codes.**

The observed trend for smaller parasperm at higher levels of sperm competition allows us to test other hypotheses of parasperm function related to postcopulatory sexual selection. The hypothesis that parasperm are produced to enhance males' success in cryptic female choice predicts the production of larger/more parasperm when postcopulatory sexual selection is more frequent (Holman and Snook 2006), which we did not observe. In contrast, a model of the evolution of hypothetical "soldier sperm" (e.g., displacers or killers of rival eusperm) suggested that selection would minimize the size of each soldier (Kura and Nakashima 2000). The idea that parasperm are displacers has not been experimentally tested in the *obscura* group, but our results suggest it may be worth pursuing.

The proportion of parasperm produced by males was positively related to eusperm length, meaning that species with long eusperm produce fewer eusperm and/or more parasperm. A model of the hypothesis that parasperm protect brother eusperm from spermicide in the female reproductive tract predicted that males would produce more parasperm as eusperm became more vulnerable to spermicide (Holman and Snook 2006). Increased

eusperm length could be associated with an increased probability of coming into contact with damaging enzymes in the uterus, meaning that more parasperm are required for adequate protection. This interspecific result motivates experiments to test this idea; it is easily falsifiable by showing that sperm are not killed by contact with the female reproductive tract (Holman and Snook 2006).

REPRODUCTIVE TRAIT EVOLUTION IN SPERM-HETEROMORPHIC AND SPERM-MONOMORPHIC SPECIES

The primary function of eusperm is fertilization. Accordingly, we found that eusperm length has coevolved very closely with the length of the VR, the sperm storage organ used to house sperm that are used first for fertilization (Pitnick et al. 1999). Interspecific correlations between sperm length and female reproductive tract morphology have been found previously in both vertebrate and invertebrate taxa (e.g., Briskie et al. 1997; Minder et al. 2005), including some sperm-heteromorphic insects (Presgraves et al. 1999; Morrow and Gage 2000). These patterns suggest that males may be adjusting sperm length to match evolutionary changes in sperm storage organ size and thereby maximize fertilization success. In support of this interpretation, male success in sperm competition is determined by an interaction between sperm and VR length in *D. melanogaster*, such that larger sperm are more successful inside females with larger VRs (Miller and Pitnick 2002). This effect is thought to be due to sperm of an appropriate size occupying a superior position near the proximal end of the VR, close to the site of fertilization (Miller and Pitnick 2002; Pattarini et al. 2006). We also found that the area of the spermathecae (where long-term sperm storage occurs; Pitnick et al. 1999), was positively correlated with male reproductive tract mass, just as in sperm-monomorphic dungflies (Minder et al. 2005), further indicating the extent of coevolution between ejaculate size and female sperm storage morphology.

We also found evidence that male investment in the ejaculate varies with the level of female promiscuity, as predicted under sperm competition theory. Similar relationships between testis mass/size and sperm competition have been observed in many other taxonomic groups, including primates, ungulates, amphibians, fish, and insects (reviewed in Parker et al. 1997). Males are expected to increase their ejaculatory expenditure under strong sperm competition, and larger testes and accessory glands typically produce more sperm and seminal fluid (Parker and Ball 2005). Thus, our data suggest that females exert selection on males to alter their investment in sperm and/or seminal fluid production in the *obscura* group. We also note that male reproductive tract mass increases with body mass in the *obscura* group as in other *Drosophila* (Pitnick 1996), and that monogamous *D. subobscura* males have the lightest reproductive organs in our dataset.

Testis mass and sperm length are highly correlated in sperm-monomorphic *Drosophila*, which is consistent with the idea that elongated sperm are costly to produce (Pitnick 1996). We did not find a positive relationship between male reproductive tract mass and eusperm length in the *obscura* group, suggesting that longer sperm are not very costly. Additionally, some sperm-monomorphic *Drosophila* pay for their large sperm with a protracted prereproductive maturation period (Pitnick et al. 1995; Pitnick and Miller 2000), but in this study we found no relationship between sperm length and age at reproductive maturity. Female investment in elongated VRs comes at the cost of delayed egg-to-adult development and increased mortality in *D. melanogaster* (Miller and Pitnick 2003). However, we found no relationship between VR length and age at reproductive maturity (i.e., the time between eclosion and reproductive maturity) in the *obscura* group. These contrasting results between sperm-monomorphic and sperm-heteromorphic *Drosophila* species raise two possibilities. First, the extreme length of the sperm produced by some sperm-monomorphic *Drosophila* relative to *obscura* eusperm could elicit correspondingly higher and therefore more visible costs. Second, there may be crucial differences in the economics of sperm production between sperm-monomorphic and sperm-heteromorphic taxa.

Assuming that parasperm are cheaper to produce than eusperm, sperm-heteromorphic species can hypothetically increase both eusperm length and overall sperm numbers without diverting additional resources to sperm production. They could accomplish this by (1) allocating a greater proportion of the available sperm production resources to making parasperm and/or (2) lowering the cost of each parasperm, for example, by reducing parasperm size or complexity so that more parasperm can be produced (see Holman and Snook 2006). Sperm-monomorphic species do not have these options; to increase sperm length, males have to sacrifice sperm numbers (Parker 1993) or divert resources away from nonsperm traits (Pitnick et al. 1995; Pitnick 1996; Pitnick and Miller 2000), both of which have associated fitness costs. This potential difference in sperm production strategies may make it more difficult to detect costs of long sperm production in sperm-heteromorphic taxa. In essence, parasperm could allow males to decouple sperm length and number and "have it both ways," producing a numerous accessory sperm morph while maintaining large eusperm size and full nonsperm trait function.

We found that all sperm traits varied with male body mass. Larger species had shorter eusperm, shorter parasperm, and produced a lower proportion of parasperm. The *obscura* group is therefore dissimilar to other sperm-heteromorphic and -monomorphic insects. In butterflies, both eusperm and parasperm length are positively related to body size (Gage 1994), however, in stalk-eyed flies, body size is unrelated to the length of either sperm morph (Presgraves et al. 1999). In sperm-monomorphic

Drosophila there is a positive correlation between sperm length and body size (Pitnick 1996). Both sperm length and sperm number are important determinants of sperm competitive ability (e.g., Parker 1993; Gage and Morrow 2003; Snook 2005). The presence of the longest sperm in the smallest *obscura* species and the highest proportion of eusperm in the largest species is evidence that optimal sperm length and number varies with body size. Our data therefore suggest that small *obscura* species benefit most from producing relatively longer eusperm and parasperm, whereas larger species are selected to increase eusperm numbers. The positive correlation between eusperm proportion and body mass might also indicate that, assuming larger *obscura* species are more fecund (Honek 1993), males produce more eusperm when females produce more eggs.

These relationships between sperm traits and body size could alternatively be mediated by a correlation between body size and the dimensions of the female reproductive tract. That is, if sperm storage organ morphology covaries with body size, a secondary correlation between sperm size or parasperm proportion and body size could arise if the female reproductive tract imposes selection on sperm traits (as observed in many studies; see above). However, our results do not support this interpretation because the dimensions of the female VR, spermathecal area, and spermathecal duct length were not significantly related to female thorax length. Female sperm storage organ size has previously been suggested to increase with body size (Gage 1994), but the results of this study and Presgraves et al. (1999) find no evidence for such a relationship. Our results indicate that sperm storage organ evolution may be more strongly affected by other variables, such as female mating frequency and ejaculate volume, compared to body size. In support of this idea, we found that spermathecal area was positively related to both female remating rate, and male reproductive tract mass.

Copulation duration was unrelated to any of the other traits, including sperm length, male reproductive tract mass, female remating rate and body size. Variation in copulation duration has been seen as an adaptation to sperm competition (for review, see Simmons 2001; Wedell et al. 2002) and may be a source of sexual conflict (Crudgington and Siva-Jothy 2000). However, our data suggest that copulation duration in this group is not related to sperm competition, the length of sperm or female behavior and morphology in this taxon.

To summarize, many of the reproductive traits we measured were correlated with one another after controlling for the confounding effects of shared ancestry. We observed patterns of coevolution that replicate findings from comparative studies of other sperm-monomorphic and sperm-heteromorphic taxa, providing further evidence for candidate universal trends in the evolution of male and female reproductive morphology and behavior. However, we also noted differences in evolutionary responses in both sperm-heteromorphic and sperm-monomorphic taxa, which may shed light on the functional significance of reproductive trait variation. This study also clarified the potential role of parasperm in the *obscura* group. Our results support previous intraspecific work showing that parasperm are not "cheap filler" and are unlikely to be "blockers" (Snook 1998a), because parasperm did not show the predicted coevolution with sperm storage organs. Our results also suggest that parasperm are not involved in cryptic female choice because we would expect investment in parasperm to be highest when postcopulatory sexual selection occurs frequently, but the reverse was found. We argue that our results are most compatible with the hypotheses that parasperm protect brother eusperm inside the female or displace rival eusperm from storage. Both hypotheses predict that parasperm should be comparatively small and cheap, and predict a positive association between eusperm length and the proportion of parasperm as we observed. Future work should investigate these hypotheses intraspecifically to illuminate the function of parasperm and elucidate the costs and benefits of infertile sperm castes.

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