

A Sterile Sperm Caste Protects Brother Fertile Sperm from Female-Mediated Death in *Drosophila pseudoobscura*

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Summary

Spermicide (i.e., female-mediated sperm death) is an understudied but potentially widespread phenomenon that has important ramifications for the study of sexual conflict, post-copulatory sexual selection, and fertility [1, 2]. Males are predicted to evolve adaptations against spermicide, but few antispermicidal mechanisms have been definitively identified. One such adaptation may be the enigmatic infertile sperm morphs or “parasperm” produced by many species, which have been hypothesized to protect their fertile brother “eusperm” from spermicide [2, 3]. Here, we show that female *Drosophila pseudoobscura* reproductive tracts are spermicidal and that the survival of eusperm after exposure to the female tract is highest when males produce many parasperm. This study clarifies the adaptive significance of infertile sperm castes, which has remained elusive in *Drosophila* and other taxa despite much recent interest [2–8]. We suggest that spermicide and male countermeasures against it are more common than is appreciated currently and discuss how spermicide could drive the evolution of several key male traits, including sperm size and number.

Results and Discussion

Males of the *Drosophila obscura* group are sperm heteromorphic; that is, they produce two distinct morphologies of sperm, short and long. The long sperm fertilize ova and are called eusperm [9], whereas the shorter parasperm type is fertilization incompetent [10, 11] and comprises 34%–94% of the ejaculate among species [3]. Parasperm are likely to be adaptive [3] but do not function to provision the female [12] or delay her remating [3, 4]. Sperm heteromorphism occurs in insects, mollusks, vertebrates, and other taxa, but its evolutionary significance remains a conundrum [8]. Parasperm were suggested recently to be a male counteradaptation to spermicide, i.e., female-mediated sperm death, protecting their “brother” eusperm in the female reproductive tract [2]. Game theory models have demonstrated that sperm heteromorphism could indeed have evolved to maximize postmating sperm survival, assuming parasperm are cheaper to produce and spermicide is substantial [2].

The current study empirically tested two key predictions of the antispermicide hypothesis for the evolution of parasperm using the fruitfly *Drosophila pseudoobscura*: (1) that the female reproductive tract is spermicidal and (2) that the infertile parasperm protect brother fertile eusperm from spermicide. Spermicide is defined here as female-mediated sperm death

resulting from chemical damage (e.g., by enzymes, adverse pH, or the immune system) or phagocytosis [1, 2] and is not necessarily adaptive to the female [2]. We tested each prediction by using both in vivo and in vitro techniques to quantify sperm viability and its relationship to parasperm production.

To test whether female *D. pseudoobscura* are spermicidal, we first measured sperm viability (the proportion of live sperm) in an in vivo experiment with natural matings. Mated females were dissected 30 min after the cessation of copulation to measure the viability of the sperm in the uterus. As a control, females were dissected immediately after mating, and then the sperm were stored for 30 min before viability scoring. We found that eusperm viability was significantly lower when sperm were left inside the female (Figure 1A; $F_{1,27} = 20.34$, $p < 0.0001$, effect size 95% confidence limits: $r = 0.39$ – 0.79). In contrast, parasperm viability was unaffected by exposure to the female (Figure 1A; treatment was absent from the minimum adequate model of parasperm viability). Eusperm viability also was lower than parasperm viability, irrespective of when sperm were removed from the female (Figure 1A). Copulation duration had no significant effect on eusperm or parasperm viability and was excluded from the minimum adequate models.

We also used an in vitro approach to test whether the female reproductive tract caused sperm death by measuring the effect of different female tissues on sperm viability [13]. Sperm from male seminal vesicles ($n = 30$) were incubated with extracts prepared by grinding and centrifuging (1) female reproductive tract tissue, (2) thoracic muscle tissue, or (3) saline. Sperm viability was measured after either 30 min or 60 min incubation by staining and counting sperm as before. The three solutions had significantly different effects on sperm viability (Figure 1B; Table 1). Eusperm viability was significantly lower when exposed to female reproductive tract extract rather than muscle tissue ($t_{132} = 4.57$, $p < 0.0001$, $r = 0.21$ – 0.50), and muscle tissue reduced eusperm viability more than did exposure to saline ($t_{132} = 6.54$, $p < 0.0001$, $r = 0.36$ – 0.60). Similarly, parasperm viability was higher after exposure to saline rather than muscle tissue ($t_{132} = 2.85$, $p = 0.005$, $r = 0.07$ – 0.39); however, there was no strong difference between the female reproductive tract and the muscle tissue solutions ($t_{132} = 1.78$, $p = 0.077$, $r = -0.02$ – 0.31). The viability of both eusperm and parasperm was lower when sperm were incubated for 60 min compared to 30 min (Figure 1B; Table 1). There were no interactions between treatment and time, indicating that the effects of the treatment solutions on sperm viability were consistent at both time intervals. Eusperm viability again was markedly lower than parasperm viability (Figure 1B).

The results of both experiments strongly suggested that the reproductive tract of female *D. pseudoobscura* causes sperm death. Therefore, we proceeded to test whether parasperm alleviate the effects of this spermicide on eusperm as hypothesized [2, 3]. In addition to measuring sperm viability in the in vivo and in vitro experiments described above, we also measured the proportion of parasperm produced by each male. If parasperm protect eusperm from female spermicide, then parasperm proportion should be related positively to eusperm

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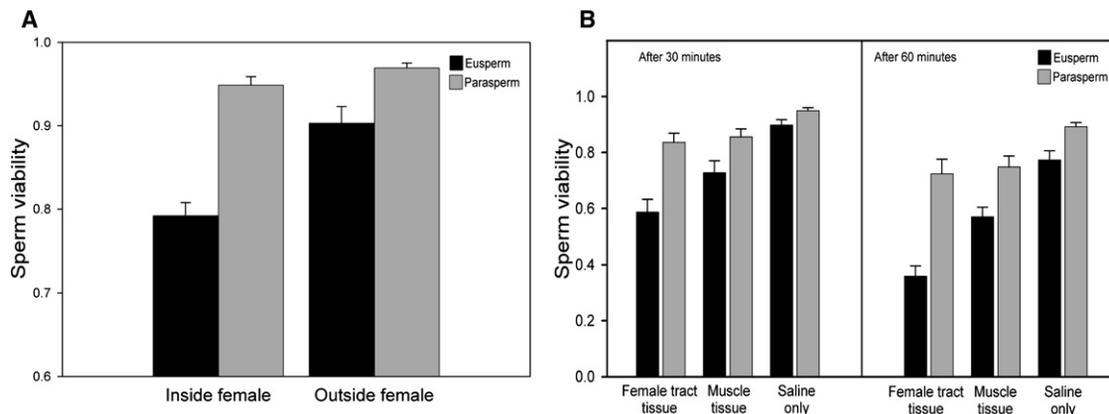


Figure 1. Mean Eusperm and Parasperm Survival after In Vivo and In Vitro Exposure to the Female

(A) In the in vivo trial, sperm were either left inside a live female for 30 min after insemination or removed from the uterus immediately after mating and stored for 30 min (n = 14 inside, n = 15 outside).

(B) In the in vitro trial, sperm were exposed to extracts containing female reproductive tract tissue, thoracic muscle, or saline only. Sperm viability was measured after both 30 and 60 min (n = 30 males). Error bars represent 1 SEM.

viability after exposure to the female reproductive tract. We also determined the mean length of eusperm and parasperm produced by each male in the in vitro experiment in order to test whether sperm length influences sperm survival as hypothesized in sperm competition theory (reviewed in [14]).

The in vivo experiment revealed the predicted positive relationship between eusperm viability and parasperm proportion after sperm had been exposed to the live female reproductive tract for 30 min (Figure 2A; $R^2 = 0.42$, $t_{11} = 2.82$, $p = 0.017$, $r = 0.15-0.84$). When sperm were removed from the female immediately after mating, no relationship between parasperm proportion and eusperm viability was observed (Figure 2B; $R^2 < 0.001$, $t_{13} = 0.07$, $p = 0.95$, $r = -0.46-0.49$), implying that the female reproductive tract may have mediated the relationship between investment in parasperm and eusperm survival. However, the design of this experiment meant that, by necessity, parasperm proportion was measured at the same time as sperm viability. Since parasperm proportion may have been altered as a result of exposure to the female, the measures of sperm survival and parasperm proportion were nonindependent. The observed results are nevertheless compelling.

The in vitro experiment did not encounter this design limitation. We were able to take subsamples of a male's sperm for

independent determination of sperm viability and parasperm proportion. The in vitro experiment also provided support for the hypothesis that parasperm protect eusperm from spermicide; we observed a significant interaction between parasperm proportion and exposure time in their effects on eusperm survival (Table 1). Eusperm viability was affected more positively by parasperm proportion in the 30 min group than in the 60 min group. We therefore tested whether parasperm proportion predicted eusperm viability after 30 min exposure to the female reproductive tract and found a strong positive relationship (Figure 3; multiple $R^2 = 0.39$, $F_{2,24} = 7.51$, $p = 0.003$; parasperm proportion: $t = 2.54$, $p = 0.018$, $r = 0.09-0.66$; latency: $t = 3.10$, $p = 0.005$), replicating the result from the in vivo assay. Also, in the in vitro trial, we found an interaction between treatment group and parasperm proportion in the model of parasperm viability (Table 1), which indicated that parasperm proportion had a more positive effect on parasperm viability when exposed to reproductive tract rather than muscle tissue ($t_{132} = 2.70$, $p = 0.008$, $r = 0.17-0.67$). Eusperm and parasperm length had no effect on either eusperm or parasperm viability in the in vitro trial (Table 1).

Taken together, our in vivo and in vitro results strongly suggest that sperm are killed by the female reproductive tract in *D. pseudoobscura* and that parasperm lessen the effects of this spermicide on eusperm viability. Although we cannot be sure that the mechanism of spermicide was the same in both experiments, we suggest that the observed sperm death was chemically mediated. The protocol of the in vitro trial involved homogenizing the female tissues and then removing all non-soluble material, strongly suggesting the presence of a soluble, cytotoxic chemical in or around the cells of the female reproductive tract. The results of the in vivo experiment are also most consistent with chemically-mediated spermicide: Phagocytosis of live sperm within the 30 min timeframe seems unlikely because the sperm are much larger than hemocytes. Also, sperm, seminal fluid and mating itself are known to induce a multitude of changes in mated female *D. melanogaster* [15–17], including elevated secretory activity [17] and upregulation of enzyme and defense genes in the lower reproductive tract [16].

Our experiments supported the hypothesis that parasperm function to protect eusperm from spermicide. In both assays,

Table 1. Two Mixed-Effect Models of the In Vitro Experiment

Sperm Morph	Model Component	F	d.f.	p
Eusperm	Treatment group	58.1	2,132	<0.0001
	Exposure time	42.9	1,132	<0.0001
	Latency	6.26	1,132	0.014
	Parasperm proportion	0.47	1,27	0.50
	Exposure time × parasperm proportion	6.43	1,132	0.012
Parasperm	Treatment group	19.2	2,132	<0.0001
	Exposure time	24.5	1,132	<0.0001
	Latency	12.2	1,132	0.0007
	Parasperm proportion	0.25	1,26	0.62
	Treatment group × parasperm proportion	4.10	2,132	0.019

These are the minimum adequate models created by backward stepwise removal of nonsignificant terms. In this way eusperm length, parasperm length, and the other interaction terms were excluded. Male identity is the random factor, n = 30 males; d.f. = degrees of freedom.

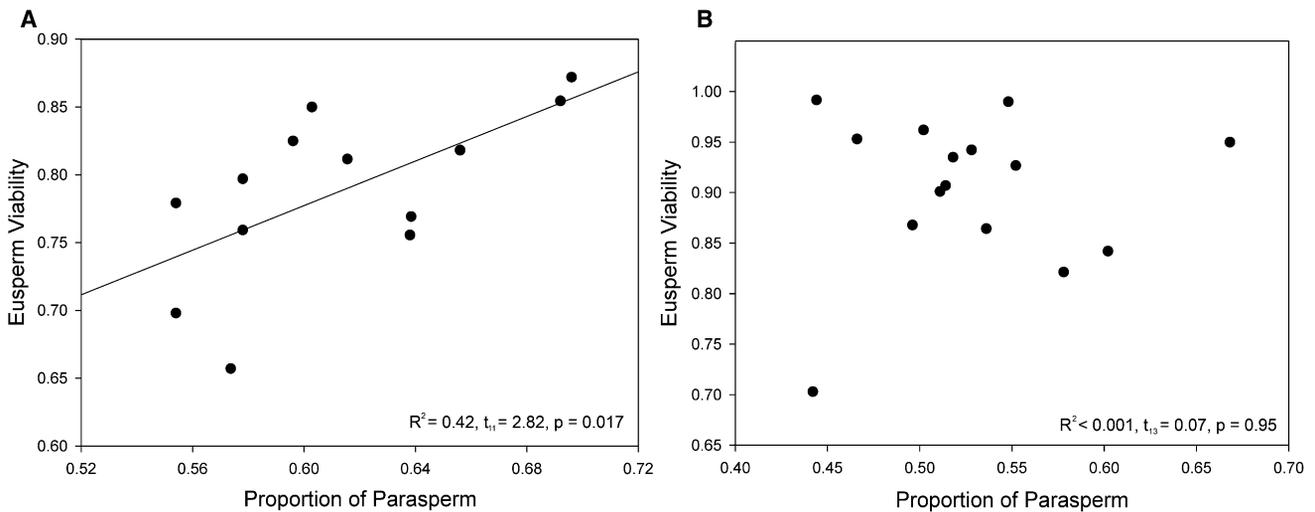


Figure 2. The Proportion of Parasperm in a Male’s Ejaculate Predicted Eusperm Viability In Vivo but Only after Exposure to the Female Reproductive Tract (A) When sperm were left inside the female for 30 min, the proportion of parasperm predicted eusperm viability (n = 13). (B) When sperm were removed from the female after mating and incubated for 30 min, there was no such relationship (n = 15).

we observed a positive relationship between eusperm viability and the proportion of parasperm after exposing sperm to the female reproductive tract. This relationship was not detected when sperm had either no (in vitro) or momentary (in vivo) contact with the female reproductive tract, implying that the female tract may have mediated this result. The precise mechanism by which parasperm protect eusperm is not clear, but we showed in a game-theory model that parasperm can increase eusperm survival simply by being present [2]. If fewer eusperm die as a result of parasperm helping to saturate the spermicidal chemicals/cells and parasperm are sufficiently cheap to produce, then parasperm do not necessarily need to perform any special function, e.g., delivering sperm-bound peptides [18] that protect eusperm. The parasperm of *D. pseudoobscura* are almost four times shorter than the eusperm, so they are likely to be less costly [3]. Parasperm might even

“buffer” the uterus of mated females to the benefit of subsequent males, as suggested for seminal fluid [19].

As well as measuring parasperm proportion, we also examined sperm length and its relationship to sperm viability. The nature of the relationship between sperm length and sperm survival is predicted to drive sperm length evolution [14, 20–22]. Although this study found no effect of length on eusperm or parasperm viability, we did find that the viability of eusperm was substantially lower than that of parasperm. Eusperm might be more vulnerable than parasperm to chemical damage, perhaps because they present a greater target. Alternatively, the lower viability of eusperm could have been an artifact; for example, eusperm may have been differentially damaged by shear forces when the coverslip was applied despite measures to prevent damaging sperm. This constraint was unavoidable because it is not possible to know if sperm

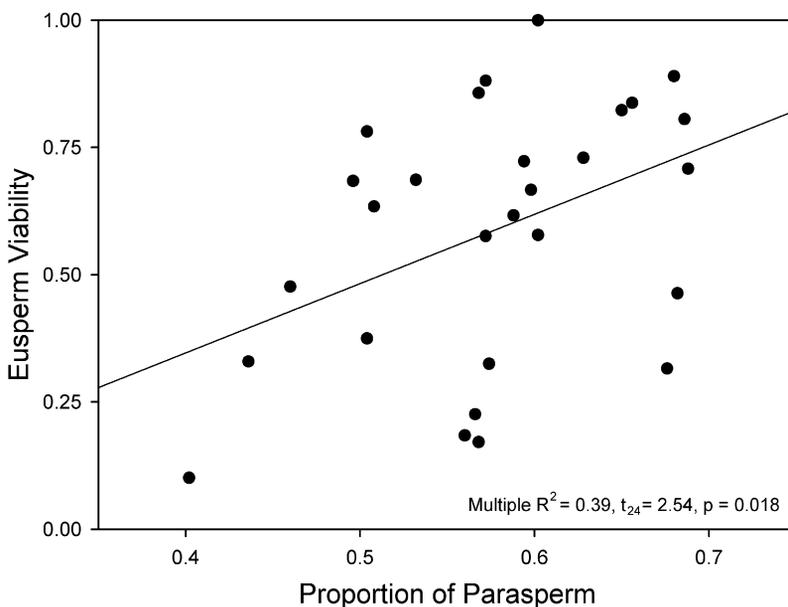


Figure 3. Parasperm Proportion Predicted Sperm Viability after 30 Min In Vitro Exposure to a Female Reproductive Tract
Statistics are from a multiple regression controlling for latency, n = 29.

were killed during staining and mounting because sperm viability cannot be directly measured in any other way [23]. However, there is no reason to think that sperm death caused by the viability assay itself occurred at a different rate among treatments, so this limitation does not diminish the reliability of our main conclusions.

Studies have indicated that spermicide may be common in insects, mammals, and other taxa (reviewed in [1, 2]), and there is growing interest in the evolutionary significance of this phenomenon (e.g., [1, 2, 13, 19, 22, 24]). Spermicide might benefit the female, for example, by providing nutriment from digested sperm or by weeding out damaged or aged sperm [1, 22, 25]. Spermicide also could facilitate cryptic female choice. For example, female *Utetheisa ornatrix* moths have a spermdigesting organ that is especially full after remating to a smaller male [24]. Alternatively, spermicide may occur as a nonadaptive by-product of other functions such as immunity [2, 25]. Whatever the function and potential adaptive significance of spermicide in females, spermicide can potentially reduce male fitness by affecting the number of viable sperm reaching storage (e.g., [20, 21, 26]). Spermicide, therefore, may be a trait over which there is sexual conflict, with the potential to produce sexually antagonistic co-evolution. A model of the evolution of antispermicidal parasperm predicted that males would escalate their investment in the antispermicidal trait if females became more spermicidal, suggesting that an arms race over sperm survival could occur [2].

There are likely to be other male counteradaptations to female spermicide in addition to sperm heteromorphism. For example, a model considering sperm-monomorphic species showed that spermicide can select for the production of additional sperm to compensate for those that die [1], so insemination of multitudinous sperm could be viewed as an adaptation against spermicide. Spermicide also might affect sperm morphology because a trade-off is thought to exist between sperm size and number [20, 21] and because different sperm morphologies might vary in their vulnerability to spermicide [1]. In support of the latter idea, this study found evidence that the long eusperm are more vulnerable than parasperm. Additionally, spermicide by the immune system could select for reduced antigenicity of sperm-cell membrane receptors.

In addition to varying the number, morphology, and biochemistry of sperm, males could mitigate spermicide by using seminal fluid, which is chemically diverse and performs a multitude of functions [27–29]. In mammals, seminal fluid contains compounds that cause immunosuppression and immune tolerance [30, 31]. Seminal fluid also contains antioxidants, which may defend sperm from oxidative damage after mating [28, 32–34], and protease inhibitors, which could ameliorate proteolytic damage [28, 29, 32, 35–37]. At least two of the seminal antiproteases of *D. melanogaster* are toxic to females [36, 37], highlighting the fascinating possibility that male countermeasures to spermicide may be a source of sexual conflict.

In summary, we found strong evidence that the reproductive tract of female *D. pseudoobscura* is a hazardous environment for sperm. Sperm death occurred after exposure to both the live reproductive tract and to an extract of soluble reproductive-tract compounds, indicating that sperm may have been killed chemically. Moreover, we twice observed a relationship between the amount of parasperm transferred and the survival of eusperm, and this relationship only was observed in the presence of the female reproductive tract. These results are consistent with the hypothesis that males produce parasperm to reduce the number of eusperm lost to spermicide.

Supplemental Data

Supplemental Experimental Procedures are available at <http://www.current-biology.com/cgi/content/full/18/4/292/DC1/>.

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