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Drosophila melanogaster seminal fluid can protect the sperm of other males

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Summary

1. Many internally-fertilizing animals produce seminal fluid which is transferred along with sperm during mating. Seminal fluid typically contains a diverse range of chemicals that coordinate sperm storage, moderate sperm motility, provide advantages in sexual selection and influence female physiology.

2. Seminal fluid is well-studied in *Drosophila melanogaster*, a species in which it has been suggested to 'incapacitate' the sperm of rival males (e.g. by killing them) and thereby provide an advantage in sperm competition. This hypothesis has been tested several times over many years, but different studies have yielded conflicting conclusions. Here, I use fluorescent staining to directly measure the effects of *D. melanogaster* seminal fluid on the survival of sperm from the same male or from a rival. The results suggest that seminal fluid improves sperm survival, even if the sperm are from a different male. This study therefore provides strong evidence that seminal fluid does not kill rival sperm, and instead can actually protect them. This study also tested whether chemicals in the female reproductive tract harm sperm as in another *Drosophila* species, but found no evidence of this.

3. These findings suggest that residual seminal fluid inside females could benefit the sperm of subsequent mates, affecting the outcome of sperm competition and influencing the evolution of ejaculates and mating systems.

Key-words: Acps, sexual conflict, sperm competition, spermicide, viability staining

Introduction

The seminal fluid of *Drosophila melanogaster* contains a diverse array of molecules that serve a multitude of functions (e.g. Wolfner 1997; Chapman 2001; Chapman & Davies 2004; Ram & Wolfner 2007). There are at least 112 accessory gland proteins (Acps) in the seminal fluid (Ram & Wolfner 2007), which are involved in sperm storage (Neubaum & Wolfner 1999; Tram & Wolfner 1999), anti-bacterial activity (Lung & Wolfner 2001a), formation of the mating plug (Lung & Wolfner 2001b) and many other processes. Seminal fluid also influences a number of female traits important to male reproductive success, such as oviposition rate and remating behaviour (Ram & Wolfner 2007), and some Acps contribute to the cost of mating in females (Chapman *et al*. 1995; Lung *et al*. 2002; Wigby & Chapman 2005). Consequently, seminal fluid is central to understanding reproduction, physiology and sexually-antagonistic co-evolution in this model species.

As well as transforming female gene expression and physiology (e.g. Heifetz & Wolfner 2004; McGraw *et al*. 2004; Heifetz *et al*. 2005; Adams & Wolfner, 2007), seminal fluid has been suggested to interfere with the ejaculates of other, 'rival', males that previously mated with the same female (e.g. Lefevre & Jonsson 1962; DeVries 1964; Harshman & Prout 1994; Price *et al.* 1999). Differences in seminal fluid composition between males could thereby explain some of the variation in second male sperm precedence, which is considerable in this species (e.g. Fiumera *et al.* 2005). The hypothesized phenomenon of sperm incapacitation (i.e. killing or any other process that renders sperm incapable of fertilization) by seminal fluid has been tested several times and has found some empirical support. Although some studies have provided evidence for sperm incapacitation, its existence has yet to be definitively demonstrated because of the difficulty of eliminating alternate hypotheses. For example, one study found that females remating to males that transferred seminal fluid but no sperm produced fewer progeny than controls, consistent with sperm incapacitation (Harshman & Prout 1994), but the stored sperm was not directly observed. This result is therefore also consistent with other processes such as 'sperm dumping' by the female in response to mating (Snook & Hosken 2004). A subsequent study expanded upon this experiment by also counting stored sperm after the second

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mating; remating to a male transferring only seminal fluid again reduced the number of first male progeny, and there was no change in the number of sperm in storage (Price *et al*. 1999). This result is consistent with incapacitation, but changes in female sperm utilization (Newport & Gromko 1984) or oviposition patterns stimulated by seminal fluid and/or the act of mating could have produced the same result. Other studies have found that the insemination of a second ejaculate containing both sperm and seminal fluid reduces the number of stored first male sperm (Civetta 1999; Price *et al*. 1999) and/or first male progeny production (Lefevre & Jonsson 1962; Scott & Williams 1993; Price *et al*. 1999). These results could be explained by sperm incapacitation (followed by disintegration, in those studies that observed sperm directly), physical displacement by the second males' sperm ('sperm displacement'; Lefevre & Jonsson 1962), sperm dumping (Snook & Hosken 2004), changes in the females' rate of sperm use, or a combination of these.

However, not all published data are consistent with sperm incapacitation. Two studies remated females to males that transfer seminal fluid only but detected no decline in progeny production relative to singly-mated controls, suggesting that the seminal fluid did not harm the resident sperm (Lefevre & Jonsson 1962; Gromko *et al.* 1984). Another study directly observed sperm in storage and used viability staining to test whether they are killed by incoming seminal fluid, and found no evidence of seminal fluid-mediated sperm death (Snook & Hosken 2004). The same study also utilized a mutant strain that transfers neither sperm nor seminal fluid to show that mating itself stimulates females to dump sperm from storage (Snook & Hosken 2004); few previous studies of sperm incapacitation are capable of distinguishing between incapacitation and dumping in response to mating. In light of these conflicting results, it is unclear whether seminal fluid detrimentally affects rival sperm in *D. melanogaster* and further study is warranted. The effects of seminal fluid on the survival of a male's own sperm also remain to be directly measured; males are predicted to produce protective seminal fluid to maximize the survival of their sperm after mating (e.g. Hodgson & Hosken 2006; Holman & Snook 2008).

Sperm might also be harmed by the female. In the congeneric *D. pseudoobscura*, some sperm die upon contact with the female reproductive tract, probably because of chemicals present there (Holman & Snook 2008). Exactly how many sperm die in the female reproductive tract in *D. melanogaster* is not known, but some pertinent data exist. Only *c.* 20% of inseminated sperm reach storage (Qazi *et al.* 2003), indicating that some sperm remain in the uterus. Some of these sperm become trapped in the gelatinous insemination mass that forms in the uterus after mating (Patterson 1947) or are pushed out during oviposition (Gilbert 1981), but it is possible that some are chemically killed by the female, preventing their entry into storage. Such female-mediated sperm death or 'spermicide' (Greeff & Parker 2000; Holman & Snook 2008) is not necessarily adaptive for the female and may occur as a by-product of other processes, such as immune defence (Holman & Snook 2008).

The present study tests a specific form of the incapacitation hypothesis, namely that sperm are killed by the seminal fluid of rival males in *D. melanogaster*. By removing confounding factors and quantifying sperm viability itself (as opposed to putative correlates such as progeny production), the experiments directly test whether seminal fluid kills rival sperm. I first measured how seminal fluid from the same or a different male affects sperm survival to test for sperm incapacitation and verify whether seminal fluid improves the survival of a male's own sperm as hypothesized. In a second experiment, I measured the effects of rival seminal fluid and female tissues on sperm survival, to test for both sperm incapacitation (via killing) and spermicide. An advantage of this experiment is that the seminal fluid had been naturally transferred to a female; some seminal fluid proteins are known to be processed soon after insemination, for example, by proteolytic cleavage and glycosylation, which can affect their function (Park & Wolfner 1995; Heifetz *et al*. 2005). Finally, I investigated sperm survival in natural matings by dissecting females to recover sperm after insemination to test whether sperm are killed by the living female reproductive tract.

Methods

INSECT CULTURES, SPERM VIABILITY ASSAY AND ANALYSIS

The wild type *D. melanogaster* used in this study were the Oregon R strain, maintained in 57 × 132 mm plastic bottles on yeasted cornmeal-agar-molasses food medium in uncrowded conditions. XO males were obtained by crossing wild type males with females from an attached XX, attached XY stock (C(1)RM, *yw*/O; Chapman 1992). These XO males do not produce sperm, and transfer only seminal fluid when mating. Additionally, *prd.res* males that lack accessory glands (Xue & Noll 2000) and transfer no sperm or seminal fluid during mating (Snook & Hosken 2004) were used. Newly-enclosed virgin flies were collected from the stocks under light CO_2 anaesthesia and matured for 5 days in yeasted, single-sex food vials.

I measured sperm viability (i.e. the proportion of sperm that are alive) using dilute LIVE/DEAD sperm viability stain (Molecular Probes L-7011; 22 μL of SYBR-14 and 44 μL of propidium iodide per 1 mL of Beadle saline, i.e. 128·3 mM NaCl, 4·7 mM KCl, 23 mM $CaCl_2$; Holman & Snook 2008), added 5 min before observation. These stains cause live sperm to fluoresce green and dead sperm red. A small proportion of sperm were stained both green and red, and these were scored as dead. I counted 200 randomly-selected sperm per sample and scored them as live or dead at ×400 magnification under UV illumination; this method was highly repeatable (10 samples were scored twice in quick succession; $r = 0{\cdot}97$, $F_{9,10} = 68$, $P < 0{\cdot}0001$). In all experiments, flies were randomly assigned to treatments, which were performed on a rotation. Different treatments were therefore administered simultaneously, preventing bias due to extraneous factors within each of the three experiments. However, the three experiments listed below were not performed simultaneously, and some of the variation in sperm viability between experiments is therefore attributable to uncontrolled factors, for example the increased skill of the experimenter over the course of the study.

Statistical tests were performed in R 2·3·2 (R Development Core Team 2006). The three experiments were each analysed with generalized linear models, using a 2-column matrix of 'number of live sperm'

and 'number of dead sperm' (made using R's *cbind* function) as the response variable and a quasibinomial error structure to account for over-dispersion. I also present 95% confidence intervals for effect size, measured by Cohen's *r*, to illustrate the range of statistically supported effect sizes (Nakagawa & Cuthill 2007; Stephens *et al.* 2007).

EXPERIMENT 1: EFFECTS OF SEMINAL FLUID FROM THE SAME OR A DIFFERENT MALE ON SPERM SURVIVAL

Oregon R males were dissected in Beadle saline to remove the reproductive organs. In the control group, the accessory glands were cut off (without piercing them) and the seminal vesicles, which store mature sperm, were separated from the testes. The seminal vesicles were then transferred to a 2·5 μL drop of Beadle saline on a microscope slide and punctured to release the sperm. The sample was then covered to prevent evaporation and left for 60 min at 21 °C, to give time for viability differences between treatments to develop (pilot dissections suggested that sperm viability is almost 100% in the seminal vesicles). I then added 1·25 μL of LIVE/DEAD stain mix and scored sperm viability. The method was the same in the other two treatment groups, except that sperm were incubated with seminal fluid from either the same male or a different male. When adding seminal fluid, both accessory glands, the ejaculatory duct and the ejaculatory bulb (all of which contribute to the seminal fluid; Ram & Wolfner 2007) were placed in the drop of saline with the sperm, punctured to the release the contents of the lumen and then lightly mixed with a pin. The control was also lightly agitated with a pin to prevent this mixing from introducing bias.

EXPERIMENT 2: EFFECTS OF PROCESSED RIVAL SEMINAL FLUID AND FEMALE REPRODUCTIVE TRACT TISSUE ON SPERM SURVIVAL

I first made three solutions with which to treat sperm. Each solution consisted of 90 pieces of female tissue (from 90 different females) in 60 μL of Beadle saline in a siliconized micro-centrifuge tube. The first treatment contained the reproductive tracts (the uterus and sperm storage organs) of 90 Oregon R females that had been mated to XO males, and therefore contained seminal fluid and female reproductive tract tissue, but no sperm. These females were left for 2 ± 0·5 h after mating before being dissected, to allow time for processing of the Acps. This delay was likely to be enough time for the proteins to be completely processed and fully capable of their natural functions (for comparison, the pre-processed forms of Acp26Aa become undetectable 30 min after mating; Park & Wolfner 1995). The second treatment contained reproductive tracts from Oregon R females mated to *prd.res* males, which transfer neither sperm nor seminal fluid (also dissected 2 ± 0·5 h post-mating). Consequently, any difference in the effects of these two treatment groups on sperm viability is due to the presence/absence of seminal fluid rather than whether or not the female mated (mating alters gene expression and stimulates secretions in the reproductive tract of female *D. melanogaster*; Begun & Lawniczak 2004; Heifetz & Wolfner 2004; McGraw *et al*. 2004; Mack *et al*. 2006). The final group was a control containing thoracic muscle tissue from mated Oregon R females cut to the same volume as a reproductive tract, as measured by graticule. After collection, the tissue was ground with a sterilized glass pestle, frozen and thawed four times and spun at 14 000 ***g*** for 10 min in a chilled centrifuge to rupture then pellet the cells. If any of the XO and *prd.res* males used in this experiment were capable of transferring sperm against expectations, this centrifugation would have removed the sperm and prevented them from biasing the experiment. The solutions were stored at –90 °C and kept on ice when outside of the freezer, and were used within two days of being made.

I dissected males and punctured their seminal vesicles in 1·25 μL saline on a glass slide, then added 1·25 μL of one of the three treatment solutions. The solutions were labelled with a code so that the sperm counts could be performed blind with respect to treatment. The drop of liquid was covered and left for 60 min at 21 °C, then 1·25 μL of LIVE/DEAD stain was added and sperm viability was scored as before. A noteworthy feature of experiment 2 is that the males supplying seminal fluid were a different genotype to those supplying it in experiment 1 (and also a different genotype to the males supplying the sperm); the mechanism by which rival sperm could be recognized and incapacitated by seminal fluid remains unknown, but it is conceivable that genotypic differences between the competing males are important.

EXPERIMENT 3: SPERM SURVIVAL INSIDE THE FEMALE REPRODUCTIVE TRACT

To assess whether recently transferred sperm die after contact with the reproductive tract of a live female, I first mated pairs of virgins and recorded copulation duration to the nearest minute. In the control group, females were dissected immediately after mating to recover the sperm from the reproductive tract. The uterus was removed by pulling on the ovipositor, washed in a drop of saline to remove loose tissue and haemolymph and then ruptured in 2·5 μL of saline to release the sperm; all visible pieces of the female tract were then removed. This sperm/saline mixture was covered and left for 60 min before assessing sperm viability as previously. In the experimental group, females were left intact for 55 min after mating before being dissected to remove the sperm, which were then counted after allowing 5 min for staining. This design ensured that sperm in both treatment groups had undergone ejaculation, been mixed with seminal fluid and were dissected out of the same organ, preventing these factors from confounding the results.

Results

EXPERIMENT 1: SEMINAL FLUID IMPROVES SPERM VIABILITY IRRESPECTIVE OF ITS SOURCE

Seminal fluid was found to affect sperm viability (Fig. 1). More sperm died when seminal fluid was not present relative to when seminal fluid from the same male (model contrast: $t_{87} = 4{\cdot}77$, $P < 0{\cdot}0001$, $r = 0{\cdot}28$–$0{\cdot}59$) or a rival male ($t_{87} = 3{\cdot}97$, $P < 0{\cdot}0001$, $r = 0{\cdot}20$–$0{\cdot}54$) was applied. However, there was no difference in sperm viability after treatment with either rival or self seminal fluid ($t_{87} = 1{\cdot}19$, $P = 0{\cdot}26$, $r = -0{\cdot}08$–$0{\cdot}32$). Sperm viability was high in this experiment relative to experiments 2 and 3 (Figs 1–3), but the three experiments were not performed simultaneously and this disparity is therefore not necessarily biologically relevant.

EXPERIMENT 2: RIVAL SEMINAL FLUID IMPROVES SPERM VIABILITY

The tissue solutions had significantly different effects on sperm viability (Fig. 2). Sperm viability was higher after treatment with seminal fluid and female reproductive tracts

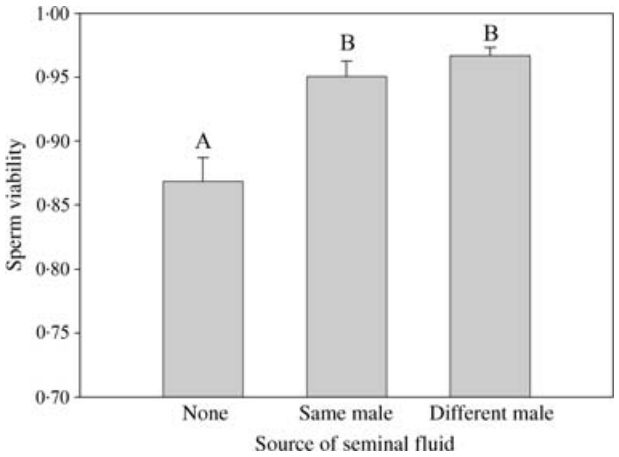

Fig. 1. Sperm viability 1 h after removal from the seminal vesicles is improved by the addition of seminal fluid, even if the seminal fluid is from a different male. Shared letters indicate that two bars are not significantly different, and error bars show 1 SE ($n = 30$ per treatment). All males were Oregon R strain.

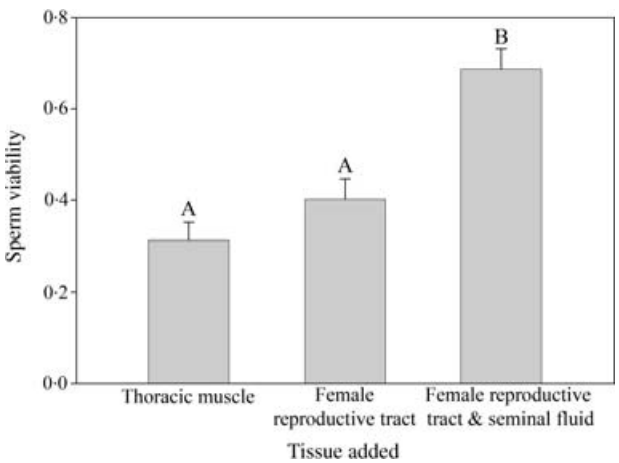

Fig. 2. A solution prepared from female reproductive tracts and seminal fluid improved sperm viability relative to solutions prepared from female reproductive tracts without seminal fluid, or thoracic muscle tissue. Shared letters indicate that two bars are not significantly different, and error bars show 1 SE ($n = 30$ per treatment). The males supplying the sperm and the females were Oregon R strain, and the males providing seminal fluid were C(1)RM, *yw*/O strain.

compared to female tracts alone ($t_{117} = 4{\cdot}22$, $P < 0{\cdot}0001$, $r = 0{\cdot}18$–$0{\cdot}50$). However, the thoracic muscle and female reproductive tract solutions killed similar numbers of sperm ($t_{117} = 1{\cdot}52$, $P = 0{\cdot}13$, $r = -0{\cdot}04$–$0{\cdot}31$).

EXPERIMENT 3: NO EVIDENCE THAT THE FEMALE REPRODUCTIVE TRACT HARMS SPERM

Treatment did not affect sperm viability; similar numbers of sperm were alive after brief or prolonged exposure to the female reproductive tract (Fig. 3; $t_{36} = 0{\cdot}81$, $P = 0{\cdot}42$, $r = -0{\cdot}19$–$0{\cdot}42$). Copulation duration also had no effect on sperm viability ($t_{36} = 0{\cdot}51$, $P = 0{\cdot}62$, $r = -0{\cdot}24$–$0{\cdot}38$) and there was no interaction between these two factors ($t_{36} = 1{\cdot}09$, $P = 0{\cdot}28$, $r = -0{\cdot}15$–$0{\cdot}45$).

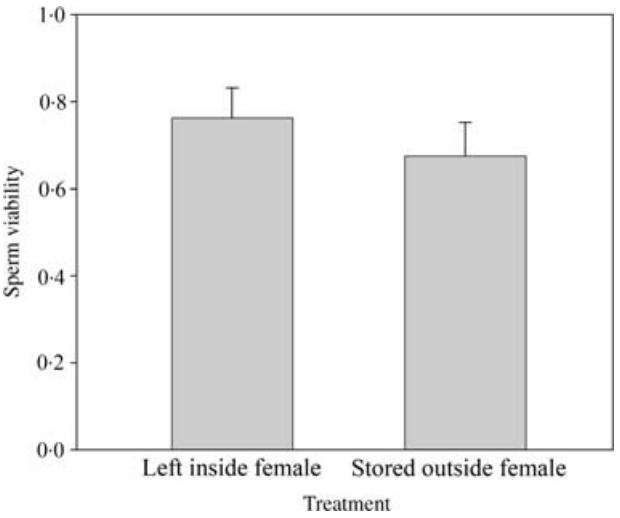

Fig. 3. Sperm viability was unaffected by a 60 min exposure to the female reproductive tract, relative to a control in which sperm were removed after mating and stored for 60 min. Error bars show 1 SE ($n = 20$ per treatment). All individuals were Oregon R strain.

Discussion

These results suggest that seminal fluid improves the survival of sperm, both before and after the seminal fluid has interacted with the female reproductive tract. Importantly, this protective function was observed even when the sperm and seminal fluid came from different males. Two different strains of males were used to supply the seminal fluid: a male from the same strain as the test male in experiment 1, and a male of an unrelated genotype in experiment 2.

The effect of seminal fluid on the viability of rival sperm was the opposite of what would be predicted if seminal fluid kills rival sperm, and hence the present study indicates that sperm incapacitation by killing does not occur. Consistent with this conclusion, three other published studies of *D. melanogaster* with the capacity to find evidence of sperm incapacitation did not find any (Lefevre & Jonsson 1962; Gromko *et al.* 1984; Snook & Hosken 2004). Additionally, last male sperm precedence in *D. melanogaster* does not differ whether the second mate is the original mate, or a different male (Gilchrist & Partridge 1995). This result suggests that if there is a sperm-killing agent in seminal fluid, it cannot distinguish between self and non-self sperm. The present study used sperm that had only recently left 5-day-old males, although it is conceivable that only sperm that have been aged inside the female are susceptible to incapacitation. However, Snook & Hosken (2004) showed that sperm removed from females 5 days after insemination were not killed by incoming seminal fluid from a rival male.

The present study demonstrates by direct observation that seminal fluid enhances sperm survival in *D. melanogaster* (this has also been shown in the ant *Atta colombica*; den Boer *et al.* 2008). Seminal fluid might protect sperm in a number of ways. For example, seminal fluid could regulate proteolysis; multiple seminal anti-proteases have been identified in *D. melanogaster* (Swanson *et al.* 2001; Lung *et al.* 2002; Mueller *et al.* 2005;

Mueller *et al.* 2007; Ram & Wolfner 2007), and deficiency in seminal anti-proteases has been linked to infertility in humans (He *et al.* 1999) and mice (Murer *et al*. 2001). Seminal fluid also contains anti-oxidants in *D. melanogaster* (Mueller *et al*. 2005), *Apis mellifera* (Collins *et al.* 2004), mammals (de Lamirande *et al*. 1997) and birds (Breque *et al.* 2003), which could protect sperm from damage by reactive oxygen species. Seminal fluid is thought to improve sperm survival after insemination by suppressing female immunity in humans and other mammals (Alexander & Anderson 1987; Aumuller & Riva 1992; Robertson 2007), which might also occur in insects (which possess innate immunity only). Finally, seminal fluid could protect sperm by buffering pH (Fordney-Settlage 1981).

The finding that seminal fluid improves sperm survival is predicted by sperm competition theory. Males are expected to possess adaptations that increase the number of live, functional sperm that reach storage or the site of fertilization (e.g. Snook 2005; Reinhardt 2007; Holman & Snook 2008) to maximize their reproductive success, especially when females mate multiply and there is an overlap of ejaculates in the female reproductive tract. In accordance with this prediction, recent studies have found that sperm viability is directly related to the outcome of sperm competition (Fry & Wilkinson 2004; Garcia-Gonzalez & Simmons 2005) and identified male adaptations that promote sperm survival (e.g. den Boer *et al*. 2008; Holman & Snook 2008). Even in the absence of sperm competition, sperm survival may have powerful effects on male fitness. The number of sperm stored from a single mating is not far in excess of the lifetime egg production of female *D. melanogaster* (Lefevre & Jonsson 1962; Qazi *et al*. 2003), implying that even a modest decrease in sperm viability could reduce male reproductive success by depriving females of sufficient sperm for full fertility (as shown in honey bees; Collins 2004). Sperm survival is emerging as an important but relatively overlooked factor that may determine the outcome of post-copulatory sexual selection and drive the evolution of multiple male and female traits (Reinhardt 2007; Holman & Snook 2008).

The present study provides empirical support for a recent hypothesis concerning sperm survival. Hodgson & Hosken (2006) proposed that males mating with previously-mated females could accrue a sperm survival or performance benefit from residual seminal fluid belonging to the previous male in the female reproductive tract. The authors speculated that this 'buffering' effect may contribute to phenomena such as last-male sperm precedence and could select for novel traits in males, such as prudent seminal fluid allocation (male reproductive potential can be constrained by finite seminal fluid reserves in insects; (Lefevre & Jonsson 1962; Baker *et al*. 2003; Rogers *et al*. 2005; Linklater *et al*. 2007). The present results demonstrate that seminal fluid can indeed improve the survival of other males' sperm.

The conclusions of the present study are dissimilar to those of Price *et al*. (1999), who conducted an investigation of sperm incapacitation and displacement in *D. melanogaster*. Their study found that when females received a normal ejaculate followed by a dose of seminal fluid (from an XO male) 7 days later, the subsequent production of offspring was reduced relative to non-remating females. Importantly, remating to the XO male did not significantly reduce the number of sperm in storage (however, statistical power was low and another study found that remating does cause resident sperm to be lost from storage, even without seminal fluid; Snook & Hosken 2004). The fact that fertility declined but the sperm remained in storage is consistent with sperm incapacitation. However, an alternative explanation for this result is that females responded to the incoming seminal fluid by using the first male sperm less efficiently (Newport & Gromko 1984) or ovipositing at a different rate; seminal fluid proteins initiate the sperm storage process (Neubaum & Wolfner 1999; Tram & Wolfner 1999) and the uterine contractions that occur during/after mating (Adams & Wolfner 2007), control the timing of oviposition (Heifetz *et al*. 2000) and affect female gene expression (Begun & Lawniczak 2004; McGraw *et al*. 2004), so it is possible that the incoming seminal fluid affected the female rather than the resident sperm. Additionally, there was no control for the stimulus of mating itself, and mating also causes contractions (Adams & Wolfner 2007) and affects gene expression (Begun & Lawniczak 2004; McGraw *et al*. 2004; Mack *et al*. 2006). Other studies have indicated that mating to males that lack both sperm and seminal fluid is enough to cause sperm loss (Snook & Hosken 2004) and a reduction in progeny production (Harshman & Prout 1994). These results imply that seminal fluid may not be required for first male progeny loss, and highlight the need to separate the effects of mating from those of seminal fluid. Finally, the experiment of Price *et al*. (1999) was repeated using three *D. melanogaster* strains, but the results were only replicated in one strain (P. D. Mack, B. A. Hammock & D. Promislow; in prep). The latter study highlights a potential limitation of most studies of sperm incapacitation, including the present article: the effects of seminal fluid on sperm could depend on male genotype. The present study demonstrated that seminal fluid protects rival sperm in two different genotypic combinations, but other crosses could have yielded conflicting results. Nevertheless, there is no known mechanism by which seminal fluid substances could recognize and incapacitate sperm based on genotypic differences between males and their sperm.

A second experiment by Price *et al*. (1999) mated females to two males: one that transferred sperm that have GFP-labelled tails, and one with normal sperm. This experiment demonstrates very clearly that first male sperm are lost from storage following remating, and that this translates into a loss of progeny (Price *et al*. 1999). However, the mechanism of this sperm loss cannot be determined, and the experiment is thus equally consistent with sperm dumping or other female-mediated effects as it is with sperm incapacitation (followed by rapid disintegration) and displacement. Additionally, the GFP males probably transferred considerably fewer sperm than their non-GFP competitors (females appear to store *c*. three times more sperm from non-GFP males than GFP males: Figs. 1b and 3b in Price *et al*., 1999). This difference in sperm number is likely to be the primary reason for the very high last male sperm precedence ($P_2 = 0{\cdot}96$) that was observed

when non-GFP males mated second, suggesting that male adaptations that directly harm or displace rival sperm, if present, are not as powerful as it would initially appear.

A recent article provided a critique of the sperm viability staining technique (Stewart *et al.* 2007). The article noted that the staining process may kill sperm, and therefore the absolute values of sperm viability must be interpreted with caution. This experimental artefact could certainly affect the sperm viability values reported here, but does not detract from the validity of the present study's conclusions. Sperm viability values were mostly high despite being measured an hour after leaving the seminal vesicles, suggesting that the assay did not kill large numbers of sperm. Potential sperm death during staining was also accounted for using similarly-handled control groups; the differences in sperm viability between treatments are of greater interest than the absolute values for the purposes of detecting incapacitation (this is also true of the other direct test of the hypothesis that seminal fluid kills rival sperm, Snook & Hosken 2004).

Another potential criticism of the present study is that the sperm used in experiments 1 and 2 were taken from the seminal vesicles and were therefore not exposed to the male's own seminal fluid. Seminal fluid might conceivably require that rival sperm are labelled with seminal fluid-derived 'markers' (which would need to vary between males) to recognize and kill them, and if this is true then my experimental design might not detect any sperm killing. However, the experiment of Snook & Hosken (2004) can eliminate this possibility; sperm in that study were inseminated naturally, and then later exposed to rival seminal fluid when the female remated; no reduction in viability was observed.

The present study was also designed to test for spermicide by *D. melanogaster* females. However, neither of the experiments involving females produced evidence that sperm are killed by the female reproductive tract. The possibility that the female tract contains harmful chemicals is not excluded by these experiments, but experiment 2 suggests that it is no more harmful than other body tissues. Experiment 3 suggests that the female reproductive tract does not cause significant sperm death in the first hour after mating. Another recent study also found that sperm viability does not decline in the first 4 days after mating in the sperm storage organs of female *D. melanogaster*, suggesting that these tissues also do not kill sperm (Civetta *et al.* 2008). The present results contrast with a recent study of *D. pseudoobscura* which found evidence of spermicide using similar experiments (Holman & Snook 2008). *D. pseudoobscura* males produce a secondary sperm morph of infertile 'parasperm' which are thought to protect the fertile sperm from this spermicide, and it is tempting to speculate that the differential toxicity of the female reproductive tracts of these species may explain why *D. pseudoobscura* possesses parasperm but *D. melanogaster* does not (Chapman 2008). However, further experiments are required to test this idea.

In summary, these results show that *D. melanogaster* seminal fluid does not kill the sperm of rival males. The possibility remains that seminal fluid inactivates rival sperm without killing them, but previous studies purporting to demonstrate incapacitation have been confounded and the mechanism for selective incapacitation of rival sperm is unclear. The available evidence suggests that sperm dumping, a change in female sperm usage patterns, sperm displacement or a combination of these causes last male sperm precedence. Seminal fluid has a significant protective effect on sperm, suggesting that it has been selected to improve sperm survival, probably to increase male reproductive success. Further studies utilizing direct observations of stored sperm and the numerous fly stocks that lack complete ejaculates will be able to differentiate between male and female influences on paternity and complete our understanding of post-copulatory sexual selection in this model species.

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