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Sperm death and dumping in *Drosophila*

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Mating with more than one male is the norm for females of many species. In addition to generating competition between the ejaculates of different males[1,2], multiple mating may allow females to bias sperm use[3,4]. In *Drosophila melanogaster*, the last male to inseminate a female sires approximately 80% of subsequent progeny[5]. Both sperm displacement, where resident sperm are removed from storage by the incoming ejaculate of the copulating male[6], and sperm incapacitation, where incoming seminal fluids supposedly interfere with resident sperm[7], have been implicated in this pattern of sperm use[5–12]. But the idea of incapacitation is problematic because there are no known mechanisms by which an individual could damage rival sperm and not their own. Females also influence the process of sperm use[13,14], but exactly how is unclear. Here we show that seminal fluids do not kill rival sperm and that any 'incapacitation' is probably due to sperm ageing during sperm storage. We also show that females release stored sperm from the reproductive tract (sperm dumping) after copulation with a second male and that this requires neither incoming sperm nor seminal fluids. Instead, males may cause stored sperm to be dumped or females may differentially eject sperm from the previous mating.

Both male- and female-mediated processes, and their interactions, contribute to differential fertilization success of males[1–3,13,14]. Males may manipulate paternity through a variety of mechanisms, including the displacement of rival sperm and incapacitation[1,15]. The physiological nature of incapacitation is unknown, but it is assumed to include either sperm inviability or sperm death[16,17]. The only data suggestive of sperm incapacitation come from experiments in *D. melanogaster*[7–9], one of the best-studied systems of sperm competition. In this species, males transfer sperm and a large number of seminal peptides (Acps) to females during copulation. These male-derived Acps have been implicated in sperm incapacitation and are therefore invoked to explain patterns of paternity[1,7–9]. In addition, although not observed directly, rival sperm have been suggested to physically displace resident sperm from storage[1,8,10,11], putatively explaining sperm-precedence patterns. But both sperm incapacitation and displacement occur within the female reproductive tract, suggesting that females may retain some control over these paternity-biasing processes, although few female-mediated processes have been demonstrated[13]. Here we test the widely held assumption that seminal fluids kill stored sperm. We find that although sperm death does indeed occur in female sperm-storage organs, Acps of rival males do not cause the death of these resident sperm. Instead, inviable sperm are a function of sperm ageing, sperm-storage effects, or direct actions by the female. We also identify a process of sperm dumping that mimics the effects of sperm displacement and is mediated by the female or by interactions between the sexes. Females dump stored sperm out of the seminal receptacle after remating, and this sperm loss does not depend on the receipt of either sperm or Acps. Sperm dumping results in fewer sperm from the first male being stored and, as a result, contributes to the precedence of sperm from the second male. Therefore, sperm incapacitation is not necessary and sperm displacement is not sufficient to explain sperm-precedence patterns in *D. melanogaster*.

To test whether seminal fluids of a rival male kill resident sperm, wild-type Oregon R (wt) females were mated once to wt males, and then four days later females were kept singly mated or remated to either wt males or spermless males that transfer only Acps (*gs1*)[18]. Twenty-four hours later, we dissected females to assess sperm viability by counting the number of live compared with dead sperm in the seminal receptacle, as this organ is believed to be the primary source of sperm for fertilization and it is only this organ in which sperm incapacitation is thought to occur[8,9]. We found a strong treatment effect ($F_{2,89} = 4.89$; $P = 0.009$) indicating that the

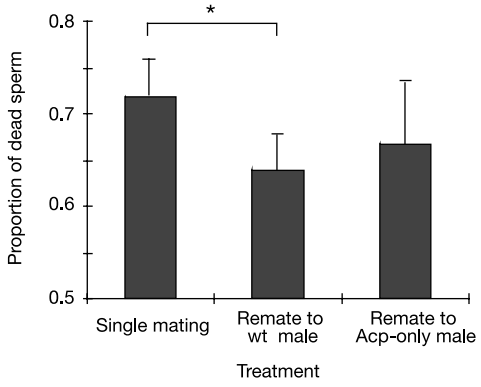

Figure 1 Effects of copulation on sperm death. The proportion of dead sperm in the seminal receptacle (mean ± s.e.m.) in females that had remated or were held as singly mated differed significantly (asterisks indicate a significant difference at the $P = 0.05$ level; see text). Sample numbers are as follows: $n = 37$ for singly mated females; $n = 37$ for females remated to wt males; $n = 21$ for females remated to Acp-only (*gs1*) males. Shown are the raw, untransformed data.

proportion of dead sperm differed across females. However, sperm death was unrelated to the presence or absence of rival male seminal fluids (Fig. 1). The proportion of dead sperm in storage did not increase when additional seminal fluid was received by females; instead, singly mated females and females remated to males transferring only Acps had the same proportion of dead sperm (Fig. 1; Fisher's protected least significant difference (PLSD), critical difference = 0.19, difference = 0.085, $P = 0.39$). This indicates that Acps have no effect on sperm viability. In addition, singly mated females had a greater proportion of dead sperm compared with wt remated females (Fig. 1; Fisher's PLSD, critical difference = 0.16, difference = 0.17, $P = 0.04$) because remated females received new viable sperm from the copulating male. Therefore, contrary to previous assumptions[7–9], incoming seminal fluid does not cause resident sperm death and, instead, sperm physiology is influenced by sperm storage rather than seminal fluid. Previous research has found that 'incapacitation' is more effective on older resident sperm[8], but how males could incapacitate rival sperm without damaging their own is unknown, especially because there is no self-sperm recognition[10]. Our results suggest that the phenomenon previously attributed to sperm incapacitation is more parsimoniously explained by increased sperm death as a function of sperm ageing, sperm-storage effects, or direct actions by females.

Females of several *Drosophila* species, including *D. melanogaster*, expel a large mass of sperm from their uterus after mating[19–21], and given that the female nervous system controls sperm storage[22], females could potentially further bias paternity by manipulating the number of stored sperm. To test whether females are involved in altering the representation of resident sperm, wild-type females were mated once to wt males, and then four days later females were either kept singly mated or remated to wt males, spermless males that transfer only Acps[18] (*gs1*), or males that copulate normally but transfer no ejaculate (*prd.Res*[23]; see Methods). Females were dissected 24 h later to determine whether sperm were present in the seminal receptacle. We found that a greater proportion of females remated to males that transferred only Acps or to those with no ejaculate lacked stored sperm compared with females remated to wt males or kept singly mated (Fig. 2; contingency table analysis, $\chi^2_{0.05,3} = 74.6$, $P < 0.001$; Tukey-type multiple comparison tests, (*prd.Res* remating = *gs1* remating) > (single copulation = wt remating)). This result was not due to first males failing to transfer sperm, because only females that had produced offspring after their first copulation were dissected. In addition, the number of offspring produced by females before their second copulation did not differ across treatments ($F_{3,119} = 0.55$, $P = 0.65$; mean progeny number ± s.e.m. for each treatment, single copulation = 83.4 ± 3.3, $n = 37$; wt remating = 78.9 ± 5.2, $n = 36$; *gs1* remating = 87.3 ± 5, $n = 20$; *prd.Res* remating = 84.2 ± 4.1, $n = 30$), indicating that differential sperm use before remating is unable to explain our result. Furthermore, we expect a random distribution of first-male ejaculate sizes with respect to treatment, because all females were mated initially to wt males, and of sperm-storage organ size, because all females were wt. Therefore, differential sperm receipt, storage and use are unlikely to explain this result. Moreover, this finding is not a consequence of either sperm displacement or sperm incapacitation, as we show here. Females remated to *prd.Res* males that fail to transfer sperm and seminal fluids receive nothing that could act as displacing and incapacitating agents, yet a large proportion of these females completely lacked stored sperm. Because males cannot directly access the female sperm stores, the mechanical act of copulation itself must induce females to dump all or some stored sperm at least sometimes, and this observation alone could explain last-male sperm precedence[5–12]. Two other studies that also used spermless males for second copulations reported that approximately 20% of females failed to produce progeny after remating[7,24], and although not directly observed, we suggest that this is the result of sperm dumping. In our study, we find that 40% of females in which we directly observed no sperm in the seminal receptacle failed to produce progeny after remating (although they produced progeny before remating). This compares with a mere 5% progeny failure in females that had at least some sperm in the seminal receptacle. Even if sperm in the seminal receptacle moved to the secondary sperm-storage organs (the spermathecae), rather than being dumped completely from the female reproductive tract, the receptacle is the organ where sperm are used first for fertilization[5]. A second male's sperm would then be the sole occupant in the primary organ, providing the mechanism for second-male sperm precedence. Furthermore, previous work has shown that males copulate for more than twice the duration necessary for complete sperm transfer[12], and that full copulation durations are required for normal second-male sperm precedence[7]. In combination with our results, these observations suggest that females dump sperm in response to copulation, and that a male must prolong copulation to elicit maximal dumping. As a mechanism influencing paternity, sperm dumping is thought to be common[3], but it has rarely been demonstrated[25–27]. Sperm dumping, either before[25] or after sperm storage[27], can be attributed to male copulatory behaviour[26,27] or cryptic female choice[3,25], or both, and could potentially also allow females to eliminate dead, useless sperm (Fig. 1) while simultaneously providing space in the fixed-volume sperm stores for new, viable sperm.

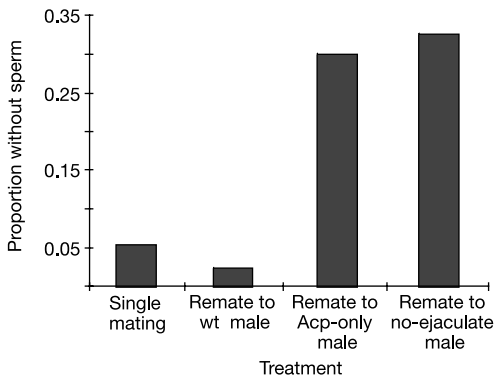

Figure 2 Sperm dumping in females. The proportion of females that had remated to spermless males that did not have any sperm in the seminal receptacle differed significantly from the proportion of females held as singly mated that had no sperm in this organ (see text). Sample numbers are as follows: $n = 3$ of 55 for singly mated females; $n = 1$ of 41 for females remated to wt males; $n = 9$ of 30 for females remated with Acp-only (*gs1*) males; $n = 15$ of 46 for females remated with males transferring no ejaculatory components (*prd.Res*).

Our findings raise two important questions: to what extent, and why, do *D. melanogaster* females dump sperm? Clearly, not every female discards all resident sperm after mating (Fig. 1), but females could discard a portion of sperm. This partial discardment would not have been detected in our experiment because the absolute number of sperm in storage was not assessed and therefore only females with no stored sperm were categorized as having dumped sperm. Moreover, some male genotypes are known to be inferior sperm displacers[9,28], and although females mediate sperm storage[22] after the receipt of seminal fluids, rates of sperm loss from storage differ when females are inseminated by different males[29]. These previous observations, together with our results, indicate that males may vary in their ability to elicit female sperm dumping. Why *D. melanogaster* females are stimulated by some males to dump sperm or to differentially eject it, but do not release sperm after copulating with other males, remains to be studied. In some species, females eject sperm from subordinate males[25], but it has also been suggested that the duration and quality of copulation can determine how many stored sperm are discarded[3,26]. It is possible that females

dump sperm to replenish their sperm stores as a consequence of sperm death due to ageing (Fig. 1), and this may be a function of male × female interactions.

Previous researchers have concluded that patterns of second-male sperm precedence in *Drosophila* can be explained by the physical displacement and incapacitation of first-male sperm[1,5–12,24]. We find that sperm dumping, in conjunction with sperm death during storage, can explain patterns of sperm use in *D. melanogaster* without invoking sperm displacement or incapacitation. In addition, there is no evidence that seminal fluids kill rival sperm. Copulation stimulates females to dump sperm, and some males are better at eliciting this behaviour than others, and/or females inherently vary in their propensity to dump sperm. These results suggest that male × female interactions are likely to be important and that females may frequently be arbiters of post-copulatory sexual selection. □

Methods

Stocks

Three fly stocks were used in these experiments. All females and some males were from the Oregon-R strain and were considered to be 'wild type' (wt). Spermless males transferring only seminal fluids were the mutant *gs1*, which lacks germ cells but retains the somatically derived accessory glands[18]. Although the exact composition of accessory gland proteins is unknown, these Acps are produced by the accessory glands and should not be affected by mutations in sperm production deriving from germ cells; it is therefore highly likely that all Acps are represented. Males transferring no sperm or Acps were generated from mutations in the *Drosophila paired* (*prd*) gene, which is normally lethal during embryogenesis, but is rescued to adulthood by two differently modified *prd* transgenes (*prd.Res*)[23]. These males have severely reduced or absent accessory glands and are sterile, because although they produce sperm, we found that they did not transfer sperm to females. We mated virgin wt females to *prd.Res* males and dissected females during ($n = 2$), immediately after ($n = 3$), 1 h after ($n = 10$), 6 h after ($n = 22$) or 24 h after copulation ($n = 17$), and in no case did we find sperm within the female reproductive tract.

Experiments

Flies were collected as virgins from mass culture, using light CO_2 anaesthesia, and housed in single-sex groups of 20 for 5–8 days before experimentation. The subsequent transfer of flies was performed by aspiration. Individual wt females were initially mated to wt males in 10 ml yeasted food vials (Vial 1). After copulation, males were removed from the vial. Four days later, females were transferred by aspiration to a new vial (Vial 2) and kept as singly mated or remated with either wt, *gs1* or *prd.Res* males. Again, after copulation, the male was removed. All matings were observed to ensure that full copulation had occurred, and Vials 1 and 2 were retained for progeny counts. The remating interval of 4 days was chosen because previous experiments demonstrating sperm displacement and incapacitation have used remating intervals of 2–7 days[7–9,12], with the average being around 4 days.

Sperm counts

Approximately 24 h after remating (or 24 h after that day for females assigned to the singly mated treatment), females were CO_2-anaesthetized and the seminal receptacle was removed from the female reproductive tract and placed on a microscope slide coated with gelatin/chrome alum[29] in 7 μl of dilute LIVE/DEAD Viability/Cytotoxicity stain (L-3224, Molecular Probes) using a modified staining protocol[30]. Sperm were released from the seminal receptacle using fine pins and incubated, in a dark moist chamber to avoid quenching the fluorescent dyes and drying the sample, for 20 min at room temperature. Sperm were then viewed at ×400 with a standard rhodamine filter using a Zeiss fluorescence microscope. Each slide was quickly surveyed to find an area of high sperm density, the position of the counted sperm mass recorded, and the number of dead sperm counted ('dead sperm at time 1'). It is difficult to see live sperm heads in a mass of tails because of the extreme length of sperm, so for each slide the sperm mass was located again and we recounted the number of dead sperm 60 min after the initial count to estimate the total number of sperm in the mass ('dead sperm at time 2') (preliminary experiments indicated that 80 min after a dissection (20 min incubation and a further 60 min), no further increases in the total number of dead sperm are seen). Sperm counts represent only a small proportion of the absolute number of sperm stored because we counted only one area of the slide. In addition, because the proportion of dead sperm will be influenced to a large degree by the total number of sperm seen (in the extreme example, if only two sperm are seen, then proportions of dead sperm can be only 0, 0.5 or 1, whereas if three are detected, then proportions of 0, 0.33, 0.67 and 1 are possible and so on), and perhaps by the sperm density/size of the sperm mass, the total sperm number in the field of view was entered as a covariate in the final analysis. This and the interaction term (total number × proportion dead) were not significant ($F < 2.6$, $P > 0.08$). Therefore, sperm number/density is unlikely to confound viability measures. All sperm counts were made blind to the mating treatment.

To verify our sampling design, we repeated counts of the same field of view for some of the samples. The number of total sperm counted each time was highly correlated (OLS regression of count 1 against count 2: $r^2 = 0.98$, $\beta = 1.0$, $F_{1,24} = 2{,}276$, $P < 0.0001$; with a repeatability of 0.995 calculated from a one-way ANOVA). We also counted two areas of some slides to confirm that no sampling biases were introduced by looking at only one sperm mass per slide. Paired *t*-tests indicated that there was no significant difference in the proportion of sperm dead on different areas of the same slide (paired-$t_{16} = -0.21$, $P = 0.83$). All data were checked to ensure that they met the assumptions of the statistical tests employed and were transformed to meet them when required.

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