Protein-specific manipulation of ejaculate composition in response to female mating status in *Drosophila melanogaster*

Laura K. Sirot[a,b,1,2], Mariana F. Wolfner[a], and Stuart Wigby[a,c,1,2]

[a]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853; [b]Department of Biology, College of Wooster, Wooster, OH 44691; and [c]The Edward Grey Institute, Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

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Female promiscuity can generate postcopulatory competition among males, but it also provides the opportunity for exploitation of rival male ejaculates. For example, in many insect species, male seminal fluid proteins (Sfps) transferred in a female's first mating stimulate increased fecundity and decreased receptivity to remating. Subsequent mates of females could potentially take advantage of the effects of the first male's Sfps and strategically reduce investment in their own ejaculate. We compared postmating responses (fecundity and sexual receptivity) of *Drosophila melanogaster* females after their first (virgin) matings (V), to the responses of females remating (M) 24 h after their first mating. The results show that M matings fail to boost fecundity and, thus, males are unlikely to gain fitness from transferring Sfps whose sole function—in V matings—is fecundity-stimulation. However, males can protect their likelihood of paternity in M matings through the transfer of receptivity-inhibiting Sfps. The levels of a fecundity-stimulating Sfp (ovulin) were significantly lower in M females relative to V females, at the same time point shortly after the end of mating. In contrast, the levels of a key receptivity-inhibiting Sfp (sex peptide) were the same in M and V females. These results support the hypothesis that males can adaptively tailor the composition of proteins in the ejaculate, allowing a male to take advantage of the fecundity-stimulating effects of the previous male's ovulin, yet maintaining investment in sex peptide. Furthermore, our results demonstrate sophisticated protein-specific ejaculate manipulation.

strategic ejaculation | male accessory gland | sexual selection | intersexual interaction | reproduction

Female sexual promiscuity creates an arena for sperm competition and other forms of postcopulatory sexual selection (1, 2). Promiscuity may also provide the opportunity for males to exploit the effects of rival males' ejaculates (3, 4). This opportunity arises because, in many species, the ejaculate not only is essential for fertilization but also can influence female postmating behavior and physiology in ways that promote male reproductive success. For example, in insects, products of the male accessory glands can have a variety of effects in the mated female, including stimulating fecundity, promoting sperm storage, and inhibiting receptivity to remating (reviewed in refs. 5–8). In mammals, functions of seminal fluids in the mated female can include stimulating ovulation, promoting sperm motility, mediating sperm storage, and protecting sperm through suppression of immune defense (9, 10; reviewed in ref. 11). If these male-induced effects persist beyond the time by which a female remates, then her next mate could exploit the effects of her earlier mates' ejaculates. A male could thereby reduce his own mating costs by decreasing investment in particular components of his own ejaculate.

Recently developed theoretical models make specific predictions about ejaculate exploitation. For example, Hodgson and Hosken (4) argue that if the ejaculate of the first male to mate with a female improves sperm survival within the female's reproductive tract, males mating with recently mated females could benefit through both increased sperm survival and decreased investment in the ejaculate components causing this effect. Similarly, game theoretic analyses predict that, under certain conditions, males mating with recently mated females should exploit the fecundity-stimulating effects of a previous male's ejaculate (3). These theoretical predictions have never been tested empirically.

Two assumptions of ejaculate exploitation hypotheses are: (*i*) males can potentially benefit from effects of the ejaculates of rival males, and (*ii*) males have the ability to adjust their production or transfer of specific ejaculate components to exploit the effects of a previous male's ejaculate. Evidence consistent with the first assumption is provided by recent empirical studies. For example, in *Drosophila melanogaster*, the seminal fluid of one male can affect both sperm survival and offspring production of rival males. Viability of sperm from one *D. melanogaster* male is higher upon in vitro exposure to seminal fluid from another male than it is in the absence of exposure to seminal fluid (12). Furthermore, the number of progeny produced by the last male to mate with a female increases when the first (spermless) male to mate transfers the seminal fluid protein (Sfp), Acp36DE (13), presumably because of Acp36DE's effect of facilitating sperm storage (14). In crickets (*Teleogryllus oceanicus*), the viability of embryos sired by one male can be enhanced by the ejaculate effects of a rival male (15). These empirical studies provide evidence that a male can potentially benefit from the effects of a rival male's ejaculate. The second assumption of the ejaculate exploitation hypothesis—that males have the ability to adjust the specific ejaculate components that they transfer to females to exploit a rival male's ejaculate—has not previously been tested.

In the present study, we tested the main prediction of the ejaculate exploitation hypothesis: that males strategically adjust specific ejaculate components in such a manner as to exploit the ejaculate effects of a rival male. We chose *D. melanogaster* as our study subject for two reasons. First, the reproductive biology of *D. melanogaster* meets the conditions under which Alonzo and Pizzari's (3) model predicts that ejaculate exploitation will occur: males have information on whether they are mating with a virgin or mated female (16) and the additional fecundity a female experiences from remating is low [at least within the first 3–6 d after the initial mating (17–19)]. Second, the functions of specific components of the *D. melanogaster* ejaculate are well-characterized (reviewed in refs. 6, 20, and 21). Of specific relevance to the current

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[1]L.K.S. and S.W. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: Lsirot@wooster.edu or stuart.wigby@zoo.ox.ac.uk.

study, fecundity is elevated primarily by two male Sfps—ovulin and sex peptide (SP)—in first matings of female *D. melanogaster* (17, 22, 23). Oogenesis is largely stalled in virgin females because of feedback-inhibition from accumulated mature oocytes in the ovary (24); ovulin lifts this blockage by causing the ovulation of these oocytes (25). Ovulin's effect on ovulation persists for only ~24 h after mating (22, 26). SP maintains oogenesis at a high level (27) and remating every 3 to 6 d is sufficient to maximize female fecundity (e.g., refs. 17–19). Thus, in matings with females that have mated recently (within 3 d), provision of additional fecundity-stimulating Sfps may be, at least partially, redundant. SP also inhibits sexual receptivity in virgin females, whereas ovulin does not (17, 22, 23); however, the fecundity and receptivity effects of SP on previously mated females have not been tested directly (28). Therefore, in this study, we first tested whether remating—and specifically SP transferred during remating—affects the fecundity and receptivity of recently mated females. We then tested whether males adjust the ovulin and SP components of the ejaculate that are transferred to females in a manner consistent with the predictions of ejaculate exploitation hypotheses.

Results and Discussion

As expected, based on previous studies (e.g. refs. 17–19), we found that remating does not elevate fecundity in recently mated (1 d earlier) females, irrespective of the presence of SP (1 d postremating, $F_{2,204} = 0.38$, $P = 0.69$; 2 d postremating, $F_{2,100} = 0.21$, $P = 0.81$; 3 d postremating, $F_{2,100} = 1.06$, $P = 0.35$; summed total of 1–3 d postremating, $F_{2,100} = 0.16$, $P = 0.85$) (Fig. 1*A*). However, female sexual receptivity is significantly reduced by remating (1 d postremating, $\chi^2_2 = 10.03$, $P = 0.007$), an effect entirely attributable to SP (SP^+ vs. no remating, $\chi^2_1 = 7.01$, $P = 0.008$; SP^+ vs. *SP0*, $\chi^2_1 = 6.78$, $P = 0.009$; *SP0* vs. no remating, $\chi^2_1 = 0.157$, $P = 0.69$) (Fig. 1*B*). Thus, when mating with recently mated females, males are unlikely to gain from transferring Sfps whose sole function is fecundity-stimulation, such as ovulin. In contrast, males mating with a recently mated female can delay future sperm competition and protect their paternity by maintaining the amount of receptivity-inhibiting SP transferred.

Based on the above findings and on ejaculate exploitation hypotheses, we predicted that males should adaptively reduce allocation of ovulin to recently mated (M) females compared with virgin (V) females. In contrast, males should maintain SP investment in M females to inhibit sexual receptivity (see above and Fig. 1*B*) and protect paternity. Using an ELISA method (29, 30), we found that ovulin levels in the reproductive tract of females shortly following M matings are significantly reduced compared with in V matings at the same time point ($F_{1,137} = 9.02$, $P = 0.003$) (Fig. 2*A*), an effect associated with reduced mating duration ($F_{1,197} = 10.47$, $P = 0.0014$) (Fig. 2*B*). However, we found no significant differences in SP levels at this time in the reproductive tracts of M versus V matings ($F_{1,168} = 0.06$, $P = 0.80$) (Fig. 2*A*). These data show that Sfps can be manipulated in a protein-specific manner. Moreover, the data support our predictions that males would reduce ovulin, but not SP transfer, to M females.

Our results suggest that second males can take advantage of first-male fecundity-stimulation, permitting a strategic reduction in ovulin allocation. SP levels are maintained, indicating that ejaculate allocation and ejaculate exploitation can be protein-specific. The mechanism by which males could adjust the molecular composition of their ejaculate is currently unclear. One plausible mechanism is through differential gene expression. Expression levels of specific Sfps vary with the perceived level of sperm competition (31). Furthermore, SP and ovulin expression varies among cell type within the male Sfp-producing accessory glands (32, 33). Moreover, at least some components of the ejaculate appear to be transferred sequentially to the female (34), so differential secretion or transfer is plausible. The ability to plastically adjust specific protein quantities in the ejaculate means that the costs to males of mating with previously inseminated females (e.g., sperm competition) may be partially ameliorated because of the second male's ability to exploit the effects of the previous male's Sfps. Thus, models of male reproductive investment (35, 36) need to be reconsidered to take into account such reductions in costs to males mating with previously mated females (3, 4).

The inability of males to further elevate fecundity in M females suggests that reduced ovulin transfer by males is adaptive because this reduction should decrease the male mating costs (37, 38) by allowing the conservation of Sfps for future mating events. A potential alternative is that a reduction in ovulin transfer [or of other fecundity-stimulating Sfps that were not quantified in this experiment (39)] is the reason why males fail to elevate fecundity in recently mated females (i.e., if males did maximize their transfer of fecundity-stimulating Sfps they might be able to further increase female fecundity). However, this explanation is unlikely given that (*i*) there would be clear fitness benefits to males in increasing female fecundity further were it possible; (*ii*) there are likely to exist physiological limits that inhibit females from exceeding a certain egg-production rate in a given environment; and (*iii*) the fecundity-stimulating effects

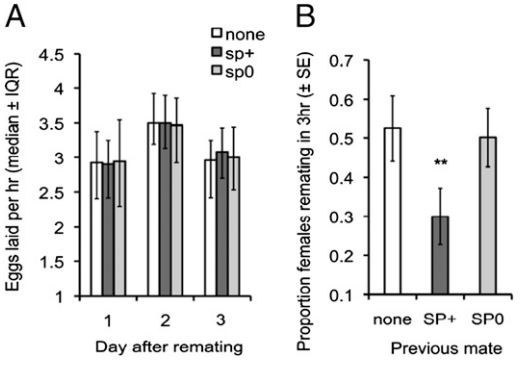

Fig. 1. Postmating responses to remating of previously mated females. (*A*) Female fecundity (eggs per hour; median ± interquartile range) over 3 d following remating (1 d after an initial wild-type mating) to a male that transfers a complete ejaculate (SP^+), an SP-null male (*SP0*) (23), or no male (none). n = 31–38 females per treatment. (*B*) Female receptivity (percentage of remating; mean ± SEM) 1 d following remating: same treatments as *A*. n = 58–80 females per treatment. $^{**}P < 0.01$.

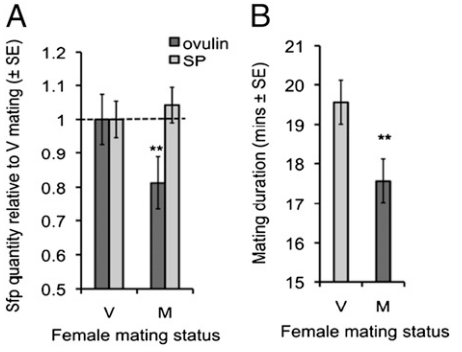

Fig. 2. Male mating investment in previously mated (M) and virgin (V) females. (*A*) Quantity of ovulin and SP transferred to M and V females (mean ± SEM). Values shown are relative to the quantity transferred to V females. n = 68–89 females per treatment. (*B*) Mating duration of M and V matings (mean ± SEM). n = 68–89 mating pairs per treatment. $^{**}P < 0.01$.

of ovulin are likely a result of the "unblocking" of accumulated mature oocytes from the ovary (25) and this will only be relevant in virgin females.

It is also possible that females could exert direct influence on ovulin levels, either by affecting the amount of ovulin transferred by males or by manipulating the fate of ovulin once it is transferred. However, several lines of evidence suggest that direct female effects are unlikely to be the major explanation for our findings. Ovulin levels in the female reproductive tract decline over time (29, 32). Some ovulin may leave the female reproductive tract and enter the hemolymph (32), but the ovulin present in the reproductive tract is proteolytically cleaved into smaller products (Fig. 3*A*) (40). Using Western blotting we found no evidence for differences in ovulin cleavage rates between V and M females: at 25 min after the start of mating, the same time point as for measurements of Sfp levels, M and V females were at identical stages of ovulin cleavage (Fig. 3*B*). Ovulin levels could potentially be affected by female "ejaculate ejection" [release of sperm and seminal fluids from the female reproductive tract after mating (41, 42)] if this had protein-specific effects. However, such ejection generally occurs much later after mating (~3 h) (41) and visual inspection of female reproductive tracts during dissections confirmed that ejaculates were present (i.e., had not been ejected) in all but 1 of the 196 samples. The single (V) sample that apparently lacked an ejaculate was excluded from further analysis. "Sperm release" [release of stored sperm from the sperm storage organs into the bursa of the reproductive tract during mating (41, 42)] would not affect our results because we use the entire reproductive tract in our ELISAs. A final possibility is that females are able to influence the amount of Sfps that a male transfers in a protein-specific manner: for example, if completion of ovulin transfer occurs later than that of SP transfer and if M females terminate ejaculate transfer earlier than V females. However, it would be difficult to distinguish male- and female-mediated effects on the amount of ovulin transferred without disabling the female to prevent her from influencing Sfp transfer, which could lead to other, unintended, effects. Thus, although potential protein-specific female-mediated effects on Sfp levels cannot be unequivocally excluded, we believe that the most parsimonious proximate explanation for lower levels of ovulin in M females is reduced male transfer.

Although mating duration was not the focus of our study, it is related to male reproductive success in *D. melanogaster*: the duration of a female's first mating is positively associated with her latency until remating (43, 44) but not with the number of sperm received (44, 45). Thus, longer first matings are associated with higher male reproductive success, presumably because of greater receptivity inhibition. However, our results demonstrate that the relationship between mating duration and receptivity inhibition is not a result of a general increase in SP transfer in longer matings, because mating duration changes independently of the amount of SP transferred (Fig. 2). We also found that the mating duration was longer for a female's first mating than for a female's second mating, results that are broadly consistent with some studies on *D. melanogaster* (45, 46) but at odds with two others (47, 48). The differences between studies in mating duration patterns may be because of differences in *D. melanogaster* strains, experimental methods, or housing conditions before the assays (43, 49). For example, the duration of a female's first mating is influenced both by exposure of the male to rival males before mating and exposure of the pair to extrapair males during mating (43, 49). It appears that in this species the relationship between female mating status and mating duration may be specific to genetic background and the particular mating environment. However, a recent cross-taxa meta-analysis showed that proxies for ejaculate investment, such as mating duration and ejaculate mass, are greater in matings with virgins than with mated females (50), a pattern consistent with our finding that mating duration and allocation of some components of the ejaculate may be reduced in M matings. Intriguingly, the same study (50) also found no evidence for increased sperm numbers transferred to virgin relative to mated females, suggesting that ejaculate investment in response to mating status may often specifically involve changes in nonsperm components, such as Sfps.

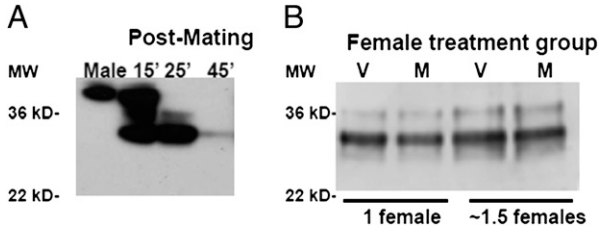

Fig. 3. Ovulin processing in mated females. (*A*) Western blot showing intact ovulin in the male accessory glands and sequential processing of ovulin in mated females with time since mating. Male lane: one-fifth of a male equivalent; female lanes: two female reproductive tracts per lane. Proteins were separated on a 10.6% SDS-polyacrylamide gel and probed with anti-ovulin antibody. Prepared by N. Buehner (Cornell University, Ithaca, NY). (*B*) Western blot showing ovulin processing at 25 min after the start of mating in reproductive tracts of females that were previously mated (M) and females that were not previously mated (V). Proteins were separated on a 12% SDS-polyacrylamide gel and probed with antiovulin antibody.

Conclusion

Across a wide range of taxa, studies have established unequivocally that males can strategically allocate sperm based on the relative risk or intensity of sperm competition (35, 51). However, only recently have researchers begun to investigate nonsperm aspects of ejaculate allocation theoretically (36) and empirically (30, 52, 53). Moreover, the idea that males can potentially exploit the ejaculates of rival males is a recent one that has previously received only theoretical attention (3, 4). Our results, together with previous studies (13), show that male *D. melanogaster* have the opportunity to exploit rival ejaculates. Furthermore, our results indicate that males may do so by tailoring the Sfp composition of their ejaculate in a protein-specific manner, suggesting an extraordinary level of sophistication in ejaculate strategies. It will now be important to determine whether such protein-specific allocation strategies are taxonomically widespread. More theoretical and empirical studies are required to determine the evolutionary consequences of such strategies for intersexual and intrasexual interactions.

Materials and Methods

Stocks. Unless otherwise specified, flies were from a Dahomey wild-type stock (28, 30). *SP0* (*sp0/Δ130*) and *SP*$^+$ (*sp*$^+$,*sp0/Δ130*) males (23) were backcrossed into Dahomey, as previously described (28).

Mating Experiments. For all experiments, fly food was supplemented by live yeast granules. Before the experiment, 10 to 20 flies were maintained in same-sex vials. Flies were 3- to 5-d-old virgins on the day of the first mating in all experiments. For all matings, females were placed with two males without anesthesia.

Effects of Mating and SP on Fecundity and Receptivity in Previously Mated Females. Day 0: females were placed individually in vials. Day 1: females were mated; after matings males were discarded. Day 2: females were transferred to individual fresh vials and given the opportunity to remate to either *SP0* or *SP*$^+$ males or they were not remated (randomly assigned). After remating, females were transferred to egg-laying vials for 24 h (1 d postremating, n = 58–80) and then either exposed to males for 3 h to measure receptivity (n = 67–81), or transferred to egg-laying vials for 2 d more (2 and 3 d post-remating) to measure fecundity (n = 31–38). For the receptivity measure-

ments, nonremating females were temporally interspersed with the other treatments to avoid time-of-day biases. The experiment was performed in two blocks. Fecundity for 2 and 3 d postmating was measured from one block only.

Sfp Quantities in V and M Females. Day 0: females were placed in individual vials and randomly assigned to M or V treatment. Day 1: M females were mated, V females were not. After matings, all females were transferred to individual fresh vials. Day 2: males were added to each female-containing vial, except for a subset of M females which were not remated to analyze background Sfps remaining from Day 1 [redesignated as Background (B) females]. Mating duration was recorded (n = 99–100 per treatment). Females were flash-frozen 25 min after the start of mating (30). Mean mating duration in this study was 18.5 min (range, 6–32 min; 5% quantile = 12 min, 95% quantile = 25 min). Pairs that mated for less than 10 min or longer than 25 min were excluded from the study. B females, which were not exposed to males on day 2, were also flash-frozen. Matings and freezings of B females were interspersed between treatments to avoid time-of-day biases. The experiment was performed in two blocks. Ovulin and SP levels in female reproductive tracts of females were measured using ELISAs (n = 68–89), as previously described (29, 30). We calculated the quantity of Sfps transferred to females as the quantity present in the reproductive tract minus the average background quantity present in B females. Using data unadjusted for background Sfps (i.e., the total quantity present from both matings in M females) would not alter our conclusions (Fig. 2*A*) that less ovulin was transferred to M females than V females and that there was no difference in the amount of SP transferred to M and V females (unadjusted data: ovulin, $F_{1,137} = 5.83$, $P = 0.017$; SP, $F_{1,168} = 1.15$, $P = 0.28$).

Ovulin Processing in V and M Females. Once in the reproductive tract of mated females, ovulin is processed into smaller cleavage products and becomes undetectable using standard methods (i.e., Western blotting, ELISA) (Fig. 3*A*) by 2 to 3 h after the start of mating (in contrast to SP, which disperses much more slowly) (29, 54). Our antibody detects cleaved ovulin, but the presence of dramatic differences in the ovulin processing rate between V and M females could potentially confound results. To check whether this was the case, we compared the rate of ovulin processing at 25 min after the start of mating. To do so, we killed females at 25 min after the start of mating by flash-freezing them in liquid nitrogen. We placed the frozen females on ice and dissected their lower reproductive tracts (including the uterus, sperm storage organs, oviducts, and parovaria) in a physiological saline. We ground the female reproductive tracts in 2× SDS sample buffer (25 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.001% Bromophenol blue) and then boiled them for 4 min. Proteins were separated on one-dimensional SDS-polyacrylamide gels. Full-length ovulin and ovulin processing products were visualized using standard Western blotting (54).

Data Analysis. Sfp transfer, mating duration, receptivity, and fecundity data from 1 d postmating were analyzed using linear mixed-effects models in R. Receptivity data were analyzed using generalized linear mixed-effects models, specifying a binomial distribution. Mating treatment was the fixed effect (SP^+, $SP0$, or none for the first experiment and M or V for the second experiment). For Sfp data, ELISA plate nested within block was the random effect. For other analyses, block alone was the random effect. For post hoc comparisons, Tukey tests in mixed-effects models used the multcomp package in R. Fecundity data from 2 and 3 d postmating were analyzed with one-way ANOVAs. Extreme outliers (Grubb's test, $P < 0.001$) were excluded from further analysis (1 M datapoint for mating duration, 1 V and 1 M datapoint for ovulin data). Fecundity and mating duration data were Box-Cox transformed to improve normality.

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