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Sex Peptide Causes Mating Costs in Female *Drosophila melanogaster*

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Summary

Conflicts between females and males over reproductive decisions are common [1]. In *Drosophila*, as in many other organisms, there is often a conflict over how often to mate. The mating frequency that maximizes male reproductive success is higher than that which maximizes female reproductive success [2]. In addition, frequent mating reduces female lifespan and reproductive success [3], a cost that is mediated by male ejaculate accessory gland proteins (Acps) [4]. We demonstrate here that a single Acp, the sex peptide [5] (SP or Acp70A), which decreases female receptivity and stimulates egg production in the first matings of virgin females [6, 7], is a major contributor to Acp-mediated mating costs in females. Females continuously exposed to SP-deficient males (which produce no detectable SP [6]) had significantly higher fitness and higher lifetime reproductive success than control females. Hence, rather than benefiting both sexes, receipt of SP decreases female fitness, making *SP* the first identified gene that is likely to play a central role in sexual conflict.

Results and Discussion

In many species, there is a potential for disparity in the optimum mating frequency of males and females. Selection for frequent matings is predicted to be stronger in males than in females; males gain fitness from each extra mating they obtain, whereas female fitness gains may cease [2] and then reverse (e.g., [3]) as mating frequency increases. Hence, the presence of female mating costs may reflect sexual conflict over mating [8]. In such conflicts, males may evolve traits that increase their fitness relative to other males but that decrease the fitness of the females with which they mate or attempt to mate.

In *Drosophila melanogaster*, the proximate mechanism underlying mating costs in females has been explored. Females that mate at high frequencies suffer fitness costs (reduced longevity and reproductive success) [3] as a result of the actions of male seminal fluid accessory gland proteins (Acps) [4]. This Acp-mediated mating cost is potentially large and is incurred in addition to reproduction costs, such as those that result from egg production [9, 10] and other nonmating activities [11]. Acps mediate a variety of effects that benefit males; such effects include stimulation of female egg production [6, 7, 12–14], reduction of female receptivity [6, 7, 14], ensuring effective sperm storage [15, 16], and promotion of male success in sperm competition [17, 18]. The female mating cost that arises from Acp transfer by males may be a side effect of Acp function [4, 19] or a direct effect that is selected to reduce the likelihood of female re-mating and/or to increase current investment in reproduction [20, 21].

We investigated whether a single Acp, the sex peptide (SP or Acp70A [5]), is responsible for mating costs in females. SP decreases female receptivity and stimulates egg production following the first matings of virgin females [6, 7]; it was generally assumed to benefit both sexes by acting as a signal to initiate high reproductive rates in successfully mated females and as a mechanism for increasing paternity in males. We exposed wild-type females throughout life either to SP-knockdown males (which produced no detectable SP [6]) or to control males (which were matched for autosomal genetic background [6]). We used two independent replicate pairs of SP-knockdown and control male lines (SP1 knockdown and C1, and SP2 knockdown and C2; see the Supplemental Experimental Procedures available with this article online). One hundred and ten females for each treatment of each line were kept in groups of five, and five males were added to each group. We measured female survival, and we sampled female mating frequency, egg production, and egg-adult viability throughout the experiments. We used female survival and age-specific offspring-production data to calculate fitness (an index of r, the intrinsic rate of population increase [22]) for each treatment of each line, and we also calculated indices of lifetime egg production per female and lifetime offspring production per female (see Supplemental Experimental Procedures).

We predicted that females continuously exposed to SP-knockdown males would mate significantly more frequently than females continuously exposed to control males because SP-induced receptivity inhibition would be absent in mates of SP-knockdown males [6, 7]. We predicted that this difference in mating frequency would lead to higher survival mating costs in females mated to SP-knockdown males, provided there was a difference in mating frequency of a least 2.2-fold (previously shown to be sufficient to cause mating costs in females [3]) over that of the control females. We also predicted that females exposed to SP-knockdown males would produce fewer eggs than controls because SP stimulates egg production in first matings of virgin females [6, 7]. To check that the survival of females continuously exposed to males was determined by male-derived reproductive costs, we measured the survival of females exposed to SP-knockdown or control males for just 48 hr.

Mating Frequency and Female Survival

Our results showed that, as expected, females continuously exposed to SP-knockdown males mated signifi-

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Table 1. Total Number of Mating and Courtship Opportunities Taken or not Taken under Continuous Exposure to SP-Knockdown or Control Males

	Mating Frequency			Courtship Frequency		
	Opportunities Taken	Opportunities Not Taken	Percent Taken	Opportunities Taken	Opportunities Not Taken	Percent Taken
SP knockdown 1	154	671	18.67%	1266	6984	15.35%
Control 1	9	724	1.23%	721	6609	9.84%
SP knockdown 2	180	605	22.93%	962	6888	12.25%
Control 2	14	747	1.84%	568	7042	7.46%

Females exposed to SP-knockdown males mated significantly more frequently than did females exposed to control males (line 1: 15.2-fold difference, $\chi^2_1 = 126.0$, $p < 0.0001$; line 2: 12.5-fold difference, $\chi^2_1 = 156.6$, $p < 0.0001$). Females exposed to SP-knockdown males were also courted significantly more often than were control females (line 1: 1.56-fold difference, $\chi^2_1 = 105.9$, $p < 0.0001$; line 2: 1.62-fold difference, $\chi^2_1 = 99.5$, $p < 0.0001$).

cantly more often than females continuously exposed to control males (Table 1). Females continuously exposed to SP-knockdown males were also courted significantly more often than control females (Table 1). However, despite mating more than 12 times as frequently and receiving significantly elevated levels of courtship, females continuously exposed to SP-knockdown males did not have reduced survival in comparison to controls (contrary to the prediction that substances other than SP cause mating costs). Instead, we found that mates of SP-knockdown males lived at least as long as, or even significantly longer than, females continuously exposed to control males [median survival, in days, from the first day of exposure to males (lower quartile, upper quartile): SP1 knockdown = 24 (21, 31), control 1 = 22 (18, 28), $\chi^2_1 = 4.35$, $p = 0.037$; SP2 knockdown = 24 (21, 29), control 2 = 24 (19, 29), $\chi^2_1 = 0.63$, $p = 0.43$]. The difference in mating rates between females exposed to SP-knockdown males and those exposed to control males far exceeded that previously shown to cause female survival mating costs [3]. Our results therefore indicate that, in terms of female survival, matings with SP-knockdown males were largely free of mating costs. Females exposed to control males mated at a lower frequency (percentage of mating opportunities taken: C1 = 1.2%, C2 = 1.8%; see Table 1) than was observed in similar assays of mating frequency in a previous study of female mating costs ("low-mating" = 2.5%, "high-mating" = 5.4%, [3]). This would have led to relatively low mating costs in our control females; however, females mated to SP-knockdown males mated at much higher frequencies (percentage of mating opportunities taken: SP1 knockdown = 18.7%, SP2 knockdown = 22.9%; see Table 1) than did the high-mating females from the previous study, in which significant mating costs were observed [3]. Hence, our chances of detecting survival mating costs in females exposed to SP-knockdown males, had such costs been present, were maximized. Of course, survival measures alone do not necessarily indicate the existence of reproductive costs, and to address whether SP contributes to Acp-mediated mating costs, we considered survival together with reproductive success (see Fitness and Lifetime Reproductive Success below).

Egg Production and Egg-Adult Viability

In further contrast to our predictions, females continuously exposed to SP-knockdown males had significantly higher egg production in the first three (line 2) or in two of the first three (line 1) of the nine egg-production samples taken during the experiments (Figure 1A). Furthermore, females continuously exposed to SP-knockdown males had marginally significantly higher indices of lifetime egg production than control females (Figure 2B). Previous work has shown that virgin females that were mated for the first time to SP-knockdown males show significantly lower egg production than females that were mated once to control males [6, 7]. We therefore did not expect to find significantly higher early egg production in females continuously exposed to SP-deficient males in this study. This observation is not attributable to a low stimulation of egg production in females mated to the control males. The same control male genotype stimulates egg production more than that of the SP-knockdown males both after single matings [6] and in assays in which males and females are housed in individual pairs (S.W., A. Crossman, and T.C., unpublished data). The increased early egg production in females continuously exposed to SP-knockdown males is consistent with a gene × mating frequency interaction. At low mating frequencies, the receipt of other ovulation- and oviposition-stimulating seminal fluid proteins, such as Acp26Aa [12] and possibly Dup99B [14], may be insufficient to offset the lack of SP, leading to low egg production in mates of SP-knockdown males. However, at higher mating frequencies the receipt of Acp26Aa and Dup99B may be at a level sufficiently high enough to result in increased egg production relative to that of control females (which receive lower levels of these other Acps). This is consistent with functional redundancy among Acps that stimulate egg production. An alternative explanation is that the higher egg production in females continuously exposed to SP-knockdown males is the result of an improvement, arising from the absence of SP, in female health. Because egg production is known to contribute to reproductive costs [9, 10], the finding that the magnitude of differences in egg production was lower and occurred over a shorter time in line 1 than in line 2 (Figure 1A) might explain why females continuously exposed to SP-knockdown males lived significantly longer than their controls in line 1 but not line 2. The eggs laid by females mated to males of both lines generally showed no differences in egg-adult viability, although mates of SP-knockdown males had significantly higher egg-adult viability in one of the later samples of the experiment (Figure 1B).

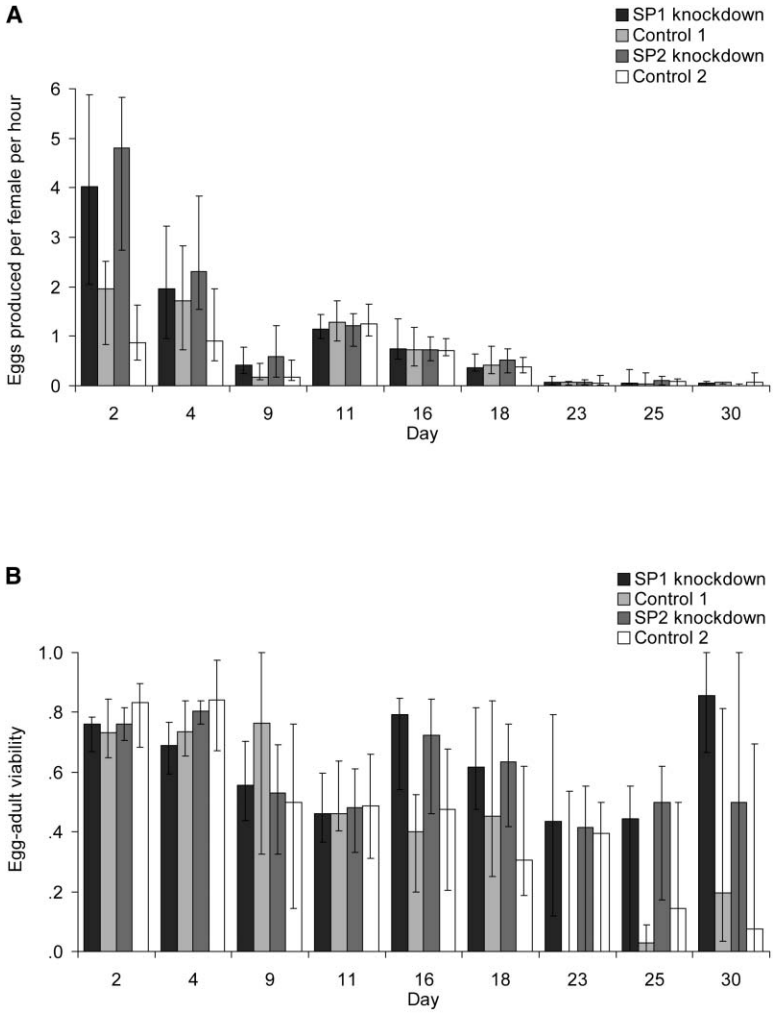

Figure 1. Age-Specific Egg Production and Viability of Eggs of Wild-Type Females Continuously Exposed to SP-Knockdown or Control Males

(A) Median (and inter-quartile range, error bars) number of eggs laid per hour by females continuously exposed to males. Females exposed to SP-knockdown males laid significantly more eggs than did females exposed to control males on days 2 and 9 in line 1 ($\chi^2_1 > 6.63$, $p < 0.01$. All other days: $\chi^2_1 < 0.84$, $p > 0.35$) and on days 2, 4, and 9 in line 2 ($\chi^2_1 > 7.50$, $p < 0.01$. All other days: $\chi^2_1 < 1.80$, $p > 0.18$).

(B) Median (and inter-quartile range, error bars) egg-adult viability for the eggs laid by the females shown in (A). Eggs laid by females exposed to SP-knockdown males had significantly higher egg-adult viability than those of females exposed to control males on day 16 in both lines (line 1: $\chi^2_1 = 14.40$, $p < 0.0001$. All other days: $\chi^2_1 < 2.65$, $p > 0.10$. Line 2: $\chi^2_1 = 4.56$, $p = 0.033$) and marginally nonsignificantly higher on day 18 in line 2 ($\chi^2_1 = 3.15$, $p = 0.076$. All other days: $\chi^2_1 < 2.08$, $p > 0.14$).

Fitness and Lifetime Reproductive Success

The most striking effect in our study was that females continuously exposed to SP-knockdown males had significantly higher indices of lifetime offspring production and fitness, as well as marginally significantly higher indices of lifetime egg production, than controls (Figure 2). Fitness (*r*) [22] was calculated from age-specific progeny and survival values. Measures based on *r* are more directly related to fitness than to lifetime reproductive success, particularly with *D. melanogaster*, which probably does much of its reproduction in expanding populations [23]. Nevertheless, the measures of lifetime egg production and reproductive success are entirely consistent with the fitness measures; they all indicate that females exposed to SP-knockdown males had higher fitness and higher lifetime reproductive success than did females mated to control males. Significant Acp-mediated survival costs of mating can be observed in females even when other costly activities, such as egg production and exposure to courting males, are held constant [4]. In this study, females exposed to SP-knockdown males had significantly higher exposure to courtship and significantly higher early egg production than did control females. Despite this, these females mated at least 12 times as often as control females and still had significantly higher fitness and lifetime reproductive success. We conclude that SP is therefore responsible for at least a major part of the Acp-mediated female mating costs in *D. melanogaster*.

Other Reproductive Costs

As expected, the survival of females exposed to males for 48 hr only was significantly higher than the survival of females continuously exposed to males for both treatments of both lines ($\chi^2_1 > 14.0$, $p < 0.0003$). The fact that females continuously exposed to males had lower survival than females exposed to males for 48 hr is likely to be due to higher reproductive costs, such as those arising from egg production [9] and the receipt of courtship [11]. In addition, other potentially harmful Acps (such as Acp62F, which reduces female survival when ectopically expressed [19]) could also contribute to reproductive and mating costs. Therefore, it is not possible to exclude the possibility that Acps other than the SP contribute to reproductive costs. As expected, because mating costs are detectable only against a background of frequent mating in this species [24], there were no differences in the survival of females exposed to SP-knockdown or control males for 48 hr in either line [median survival, in days, from the first day of exposure to

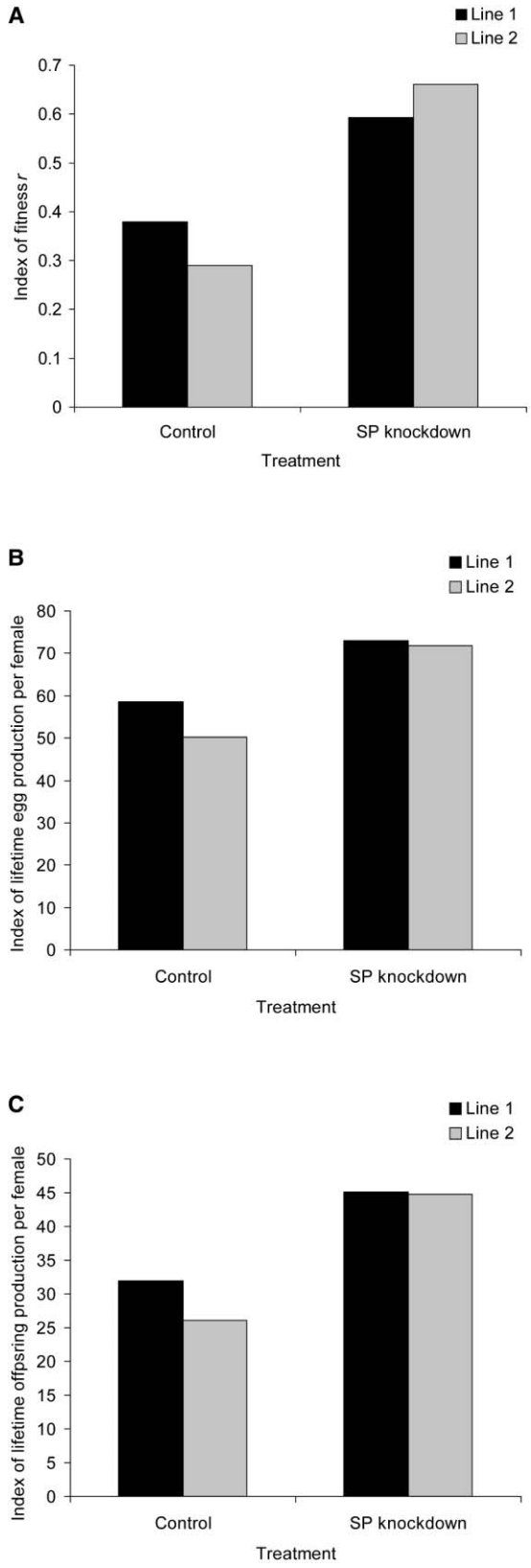

Figure 2. Indices of Fitness, Lifetime Egg Production, and Lifetime Offspring Production of Wild-Type Females Continuously Exposed to SP-Knockdown or Control Males

Indices of (A) fitness, given by *r* (the intrinsic rate of population growth); (B) lifetime egg production per female; and (C) lifetime offspring production per female for females continuously exposed to SP-knockdown or control males. The percentage increases in females mated to SP-knockdown males above those mated to control males were as follows: for fitness (*r*), 55.7% and 127.7% (lines 1 and 2, respectively); for lifetime egg production per female, 24.5% and 43.0%; and for lifetime progeny production per female, 41.2% and 71.4%. Females exposed to SP-knockdown males had significantly higher values of *r* ($F_{1,2} = 26.51$, $p = 0.036$) and lifetime offspring production ($F_{1,2} = 29.46$, $p = 0.032$) than did females exposed to control males. Females exposed to SP-knockdown males had marginally significantly higher values for lifetime egg production than females exposed to control males ($F_{1,2} = 18.15$, $p = 0.051$).

males (lower quartile, upper quartile): SP1 knockdown = 30 (24, 36), control 1 = 30 (23, 36); SP2 knockdown = 30 (22, 36), control 2 = 31 (23, 36); $\chi^2_1 < 0.01$, $p > 0.92$ both lines].

X chromosome differences between SP-knockdown and control males could have contributed to differences in male behavior (e.g., courtship and mating frequency) and hence in female reproductive success. However, differences in X chromosome constitution are not likely to confound our results through any potential effects on Acp levels because the genes encoding all the Acps responsible for mating costs in females [4] are autosomal [25]. However, we cannot exclude the possibility that there are X-linked, *trans*-acting genes that modulate Acp function (e.g., genes that encode for enzymes that regulate Acp potency).

Mating and courtship rates of the control males in our experiment are broadly comparable to the range seen in the wild-type cage populations from which the experimental females were drawn. Even in the wild, females are subject to very intense bombardment from males [26], and multiple mating is common [27]. The mating and courtship rates observed were also comparable to those seen in previous experiments [3]. If mating and courtship were artificially high in our experimental setup, the lack of cost seen in females mating with SP-knockdown males would be all the more remarkable.

SP and Sexual Conflict

Males gain from SP transfer because, even though it ultimately reduces the fitness of their mates, SP also induces a refractory period [6, 7] that significantly increases "per-mating" paternity levels (our unpublished data). Our results indicate that, rather than benefiting both sexes, the receipt of SP decreases female fitness. We would therefore predict that females with elevated SP resulting from ectopic SP-induction [28] or from matings with males that produce and transfer elevated levels of SP, should incur increased mating costs. Our results are also consistent with the finding, from a large-scale study of the effects of variation in male-sperm competitive ability on females, of positive correlations between the length of female refractoriness (i.e., re-mating interval) and early female mortality [29]. This finding may suggest that males that can induce longer re-mating intervals can impair female survival. Our study highlights SP as an obvious candidate mechanism.

Females could gain indirect genetic benefits from mating with SP-transferring males if their male offspring

had higher reproductive success. However, such benefits are likely to be small in comparison to the direct costs incurred by the receipt of SP (e.g., [30, 31]). Females could also benefit directly, through increased egg production [6, 7], from the receipt of SP if mating opportunities were limited to one or a very few matings. However, multiple mating is the norm in *D. melanogaster* both in the laboratory and in the wild (e.g., [27, 32]), and as we have shown here, fecundity benefits through receipt of SP may not occur with frequent mating. It is therefore unlikely that females often benefit from the receipt of SP. Consequently, the *SP* gene is likely to play a role in sexual conflict rather than in cooperation.

Initially, natural selection may have caused females to evolve a sensitivity to substances such as SP and allowed them to adaptively modulate egg production and receptivity after sperm transfer [33]. Our demonstration of direct costs that result from the receipt of SP is, however, consistent with a scenario in which SP is under the influence of sexual selection and sexual conflict. Such a scenario may have selected for SP activity that increased male reproductive success regardless of the effect upon females. If SP is subject to sexual conflict, then theory predicts that it should show relatively rapid evolutionary change. Although the *SP* C terminus appears relatively conserved in the melanogaster species subgroups, *D. subobscura* [34] and *D. suzukii* [35], the N terminal region is somewhat divergent [34, 36], and significant departures from neutrality have been detected in the region flanking the 5′ end of the *SP* gene [37]. It is not clear whether SP alone is responsible for female mating costs or whether harm is caused by the interaction of SP with other ejaculate molecules. SP binds to sperm and can be detected on sperm heads several days after its deposition in the female reproductive tract [7]. There is no reduction in the cost of mating in females continuously exposed to spermless males [38], which suggests that the SP that is harming females must be free from association with sperm. SP appears to stimulate egg production by causing the release of juvenile hormone (JH) BIII from the Corpora allata [39], and this release stimulates oocyte progression in the ovary [40]. Increased JH levels are negatively associated with lifespan in other insects [41]. Hence, costs, such as immunity suppression, that result from the effects of increased JH [42] are candidate mechanisms for future study.

Supplemental Data

Detailed Experimental Procedures are available with this article online at http://www.current-biology.com/cgi/content/full/15/4/316/DC1/.

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