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Evolution of female remating behaviour following experimental removal of sexual selection

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The relatively small number of ova produced by a female can be fertilized by a single ejaculate in most species. Why females of many species mate with multiple males is therefore enigmatic, especially given that costs associated with remating have been well documented. Recently, it has been argued that females may remate at a maladaptive rate as an outcome of sexually antagonistic coevolution: the evolutionary tug-of-war between manipulation by one sex and resistance to being manipulated by the other sex. We tested this hypothesis experimentally for the evolution of the female remating interval in a naturally promiscuous species, *Drosophila melanogaster*. In two replicate populations, sexual selection was removed through enforced monogamous mating with random mate assignment, or retained in polyandrous controls. Monogamy constrains the reproductive success of mates to be identical, thereby converting prior conflicts between mates into opportunities for mutualism. Under these experimental conditions, the sexually antagonistic coevolution hypothesis generates explicit predictions regarding the direction of evolutionary change in female remating behaviour. These predictions are contingent upon the mechanism of male manipulation, which may be mediated biochemically by seminal fluids or behaviourally by courtship. Levels of divergence in female remating interval across lines, and in male ejaculatory and courtship effects on female remating, were quantified after 84 generations of selection. Data refute the hypothesis that the evolutionary change in female remating behaviour was due to sexually antagonistic coevolution of courtship signal and receiver traits. The data were, however, consistent with a hypothesis of sexual conflict mediated through ejaculate manipulation. Monogamy-line males evolved ejaculates that were less effective in inducing female non-receptivity and monogamy-line females evolved to remate less frequently, symptomatic of lowered resistance to ejaculate manipulation. The consistency of the results with alternative hypotheses to explain female promiscuity are discussed.

Keywords: sexual selection; sexual conflict; remating; sperm competition; *Drosophila*

1. INTRODUCTION

In most organisms it is adaptive for males to mate with multiple females. By doing so, males are likely to increase their reproductive success. The adaptive significance of females mating with multiple males, however, is more enigmatic. Females may receive sufficient sperm in a single ejaculate to meet their seasonal, or even lifetime, fertility needs, and so mate number may not be correlated with the number of progeny produced as it is for males (Bateman 1948). Moreover, there may be substantial costs associated with remating for females (Eberhard 1996; Chapman *et al.* 1998; Johnstone & Keller 2000), including the time and energy devoted to courtship and copulation, the increased risk of predation while mating (Wing 1988; Arnqvist 1989), the risk of injury by males (Parker 1970*a*; Arnqvist 1989), the increased risk of acquiring sexually transmitted diseases or parasites (Sheldon 1993) and the harm caused by toxic seminal fluids (Fowler & Partridge 1989; Chapman *et al.* 1995; Gems & Riddle 1996; Rice 1996; Holland & Rice 1999; S. Pitnick, unpublished data). Nevertheless, it is clear that females of most species are promiscuous (Thornhill & Alcock 1983; Ridley 1988; Birkhead & Møller 1992, 1998; Andersson 1994; Eberhard 1996).

It was recently proposed that females remate at intervals that are maladaptive, as the result of an evolutionary arms race between male manipulation of female remating behaviour and female resistance to being manipulated (e.g. 'sexual dialectics' (Gowaty 1997) and 'chase-away' (Holland & Rice 1998) models of sexual selection). Application of this hypothesis to female remating stems from the recognition that mating with multiple males by females provides a ubiquitous source of sexual conflict. Female remating will be costly to the males they have previously mated with by reducing their fertilization success because they do not wait until their sperm stores are exhausted before remating (Parker 1970*b*; Birkhead & Møller 1998). However, it is nearly always in a subsequent male's best interests to copulate with females previously mated to other males. The optimum remating rates of a female, from the perspectives of herself, her previous mate and her potential future mates thus differ from one another. The result of this conflict may be sexually antagonistic coevolution between genes affecting female remating probability and genes affecting male traits that act to stimulate or inhibit female remating. Females may therefore remate more or less frequently than the rate that maximizes their fitness, depending on the outcome of this three-way evolutionary conflict (see Rice 1998, p. 264).

Determining the extent to which female remating behaviour has been shaped by intersexual conflict requires a detailed understanding of the male- and female-mediated mechanisms that influence receptivity in non-virgin females. The best-studied organism in this regard is *Drosophila melanogaster*, a naturally promiscuous

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species where females typically contain sperm from multiple males (see Harshman & Clark (1998) and references therein). Evidence suggests that males influence (e.g. manipulate) female probability of remating through their ejaculates and their courtship behaviour. Males transfer seminal fluid proteins that enter the female's circulatory system where they influence her neuroendocrine system in ways that benefit the male, including depression of the female's willingness to remate (reviewed in Wolfner 1997). In addition, a previous experiment indicates that courtship is sexually antagonistic in *D. melanogaster*. In the controlled absence of an evolutionary response by females, males evolved to increase the mating rate within a population. Female reproductive success varied inversely with mating rate through diminished female survival (Rice 1996).

We tested the applicability of the sexually antagonistic coevolution hypothesis to the evolution of female remating frequency in *D. melanogaster* by examining evolutionary divergence in this trait following experimental removal of sexual selection. In two replicate pairs of populations sexual selection was removed through enforced monogamous mating with random mate assignment, or retained in promiscuous controls. Under these experimental conditions, the sexually antagonistic coevolution hypothesis generates explicit predictions regarding the direction of evolutionary change in female remating behaviour. These predictions, however, are contingent upon divergence in male manipulation traits as outlined below.

Two opposing predictions are generated by the sexually antagonistic coevolution hypothesis regarding the consequences of monogamy selection. These predictions depend on the evolutionary dynamic in the selection lines involving conflict between females and prospective mates and between females and previous mates (Rice 1998). Prospective mates attempt to manipulate females through courtship signals (Holland & Rice 1998). Previous mates manipulate females through their ejaculates. Monogamy constrains the reproductive success of mates to be identical, thereby converting prior conflicts between mates into opportunities for mutualism. Under experimentally enforced monogamy, males have been observed to evolve a more benevolent form (i.e. reduced female mortality) and females evolve lowered resistance to male harm (Holland & Rice 1999). If males evolve a reduction in their ability to manipulate females through courtship, then females may consequently evolve lowered resistance to courtship manipulation. If this is true then monogamy-line females are predicted to remate more quickly than control-line females when courted by wild-type males unrelated to the selection lines (hereafter referred to as 'test' males). On the other hand, if males evolve a reduction in their ability to manipulate females through their ejaculate, then females may consequently evolve lowered resistance to ejaculate manipulation. If this is true, then monogamy-line females are predicted to be more refractory to remating than control-line females following insemination by test males. We employed three male order treatments (test–test, test–monogamy and monogamy–test) to determine the extent to which relevant male courtship and ejaculate traits influence the female remating interval.

2. MATERIAL AND METHODS

(a) *Experimental removal of sexual selection*

The selection lines examined in this study are the same as those reported on by Holland & Rice (1999) (see their paper for details of the protocol by which the 'monogamy' and 'control' lines were established and maintained). Briefly, from a single ancestral wild-type population of *D. melanogaster*, two replicate (A and B) pairs of lines were established, each replicate consisting of a monogamous and a control population. Every generation, 114 virgin females from each line were individually housed with one (monogamy lines) or three (control lines) randomly assigned virgin males from within that line. In all other respects all populations were treated identically. Males and females were paired for five days before being transferred to a 'culture vial' for a single day. On the day of maximum adult emergence from the culture vials, progeny from 100 productive culture vials were pooled for each line and virgin males and females were randomly selected from each pool to begin the next generation.

All traits were measured on flies reared under standard conditions by transferring, for each selection line, 50 first-instar larvae to each of three 8 dram shell vials containing 8 cm^3 of standard cornmeal–molasses–agar medium. On the day of eclosion, virgin females were collected following anaesthetization with carbon dioxide. Female and male sizes were determined in all experiments by measuring the length of the thorax. Test males were from a strain unrelated to that from which the selection lines were established. The test-male population was founded in 1996 from 50 isofemale lines collected from a Napa Valley, CA, USA vineyard. These lines were combined and maintained continuously in a large population cage supporting at least 1000 individuals.

(b) *Female remating interval*

The female remating interval was assayed following 84 generations of selection using both monogamy-line males and test males. In each of the four selection lines (two monogamy lines and two control lines), remating was assayed in each of three treatments: first, initially mated to test male, remated to test male; second, initially mated to test male, remated to monogamy-line male; and third, initially mated to monogamy-line male, remated to test male. A fully balanced design was not employed as a treatment with females initially mating and remating to monogamy-line males would not help to distinguish between the alternative hypotheses for the evolution of female remating interval. Because we are testing for coevolutionary patterns between the sexes, replicate-A monogamy-line males were used in the treatments with replicate-A females and replicate-B monogamy-line males were used with replicate-B females.

To obtain initial matings, virgin four- to five-day-old females from each line were randomly assigned to the male treatments ($n = 40$ females per treatment) and each was paired with a single, four- to five-day-old virgin male in an 8 dram shell vial containing medium and live yeast. All pairs were observed to copulate, after which the males were removed, measured and discarded. These non-virgin females were then permitted 2 h opportunities to remate on each successive day by aspirating one virgin five- to ten-day-old male of the appropriate type into their vial. All vials were examined for copulating pairs every 10 min (copulation lasts *ca.* 20 min). Following each remating, both the male and the female were measured and discarded.

Table 1. *Results of survival–regression analysis of female remating interval for each paired replicate*

variable	χ^2	d.f.	p
replicate A			
monogamy versus control female	9.82	1	0.0017
male mating order	1.23	2	0.54
female body size	0.001	1	0.98
replicate B			
monogamy versus control female	24.85	1	< 0.0001
male mating order	23.71	2	< 0.0001
female body size	4.65	1	0.031

After 2 h, males were removed from the vials of females that did not remate. This process continued for 14 days, during which time 14 out of the 480 females escaped or were accidentally killed prior to remating. Out of the remaining 466 females, 434 (93.13%) were observed to remate. During the remating interval, females were provided with fresh vials every second day. All vials were retained to quantify the number of progeny produced prior to remating.

(c) *Statistical analyses and data interpretation*

The remating intervals of females in monogamy and control lines were compared using Survival Analysis Tools for Statview (Abacus Concepts, Inc. 1994). These statistical tools are appropriate for evaluating most data consisting of the elapsed time between two events of interest. Cumulative remating curves including all females were compared using a parametric regression analysis of log-normally transformed remating intervals. Females accidentally lost or killed during the experiment and those not remating by the end of day 14 were also included in the analysis as censored data. In the initial statistical model, replicate (A, B), female selection (monogamy, control) and male order (test–test, test–monogamy, monogamy–test) were entered as factorial variables and female size was entered as a covariate to control for possible correlated effects of body-size differences (S. Pitnick, unpublished data) on remating interval. The number of progeny produced by females prior to remating was analysed with analysis of covariance (ANCOVA).

Strong evidence of the nature of responses in selection experiments is derived in part from their consistency across selection replicates. Heterogeneous responses may arise in selection replicates for a variety of reasons, including inadvertent selection, inbreeding, genetic differences between the base populations and multiple mechanisms underlying some selection responses contributing to different correlated responses (Gromko 1995; Harshman & Hoffmann 2000). Although we recognize that significant yet inconsistent evolutionary responses may provide information about character trade-offs and selection-response mechanisms, we also recognize the limited potential for making strong inferences regarding the selective basis of such traits. In this regard, the conclusiveness of the current study is severely limited by the existence of only two selection replicates.

3. RESULTS

There was no significant effect of replicate ($F_{1,455} = 0.35$, $p = 0.552$) on female remating interval but there was a significant replicate by male-mating-order interaction effect ($F_{2,455} = 4.30$, $p = 0.014$). Therefore, male effects on female remating differed between replicates, making it necessary to conduct independent analyses on the two replicates. In both replicates, control-line females remated significantly faster than monogamy-line females (table 1 and figure 1). Control-line females were significantly larger than monogamy-line females (mean ± s.e.m. thorax length for replicate A, control 0.921 ± 0.004 mm; monogamy 0.879 ± 0.004 mm, $F_{1,228} = 51.24$, $p < 0.0001$ and for replicate B, control 0.924 ± 0.003 mm; monogamy 0.895 ± 0.004 mm, $F_{1,234} = 30.66$, $p < 0.0001$) but female size did not significantly influence female remating interval in replicate A and had only a marginal effect in replicate B (table 1), with larger females remating more quickly.

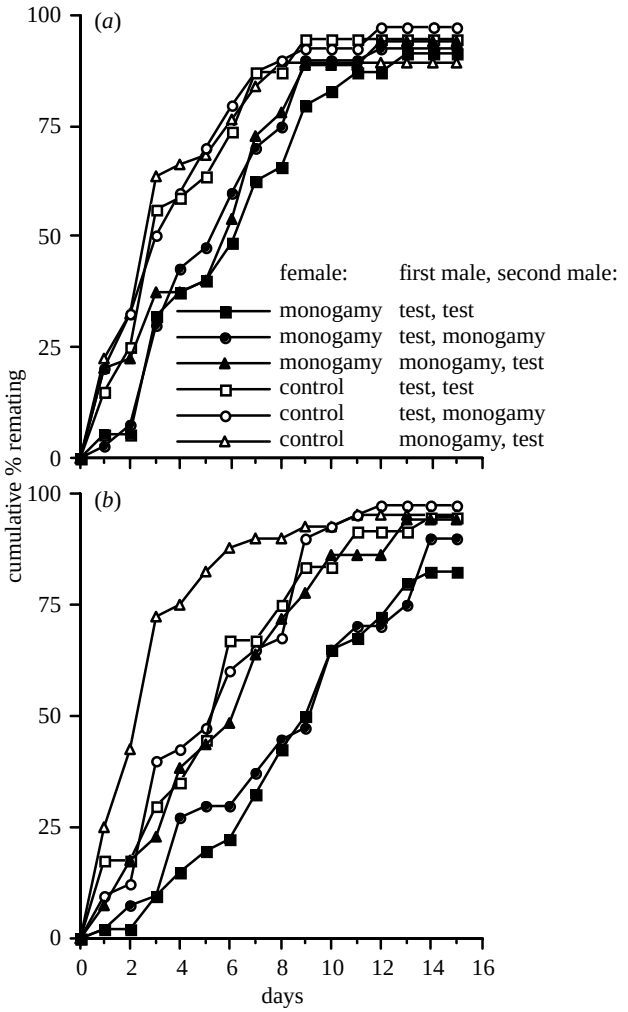

Figure 1. Cumulative per cent remating over time by monogamy- and control-line females when presented with three different combinations of males. (*a*) Replicate A and (*b*) replicate B.

There was no male-order effect in replicate A but a highly significant effect in replicate B (table 1). Moreover, these effects of male order in replicate B occurred in both monogamy- and control-line females (figure 1). Pairwise contrasts revealed that, in all cases, the male-order effect was due to a significantly shorter remating interval when the first male came from the monogamy line, and was irrespective of the identity of the second male. There were no significant differences between treatments when the

Table 2. *Summary statistics of pairwise contrasts to determine the influence of male mating order upon female remating interval*

pairwise contrasts of male mating order		χ^2	d.f.	p
replicate A				
test–test	test–monogamy	0.07	1	0.79
monogamy–test	test–test	1.12	1	0.29
monogamy–test	test–monogamy	0.67	1	0.41
replicate B				
test–test	test–monogamy	0.057	1	0.81
monogamy–test	test–test	18.66	1	< 0.0001
monogamy–test	test–monogamy	16.88	1	< 0.0001

first mates were test males and second mates were either monogamous or test males (table 2).

Although analysis of the entire data set revealed no male effects in the replicate-A lines, examination of the cumulative remating curves (figure 1) suggests that male-order effects may have been manifest early in the experiment that were not detectable later on. There is a biological expectation of this effect, as the influence of seminal fluid on female remating behaviour has been demonstrated to be transient, lasting only one to two days (Kalb *et al.* 1993). We believe, therefore, that examination of early effects will provide a more sensitive assay capable of detecting more subtle evolutionary responses. We therefore conducted a post-hoc statistical comparison of the proportion of females remating within two days using contingency-table analysis. In both replicate A and replicate B, monogamy-line females initially mated to monogamy-line males were significantly more likely to remate within two days than those initially mated to test males (replicate A: 9 out of 40 females initially mated to monogamy-line males remated versus 5 out of 80 initially mated to test males, $G_{adj} = 6.26$, d.f. $= 1$, $p < 0.025$; replicate B: 7 out of 39 females initially mated to monogamy-line males remated versus 4 out of 80 initially mated to test males, $G_{adj} = 4.88$, d.f. $= 1$, $p < 0.05$). Control-line females initially mated to monogamy-line males were significantly more likely to remate within two days than those initially mated to test males in replicate B (17 out of 40 females initially mated to monogamy-line males remated versus 12 out of 80 initially mated to test males, $G_{adj} = 10.54$, d.f. $= 1$, $p < 0.005$) but not in replicate A (12 out of 39 females initially mated to monogamy-line males remated versus 23 out of 79 initially mated to test males, $G_{adj} = 0.02$, d.f. $= 1$, $p > 0.5$).

To examine the fitness consequences of female remating behaviour for males, we examined the number of adult progeny produced by females prior to remating. Within each of the 12 experiments (three male-order treatments $\times$ two selection treatments $\times$ two replicates) there was a highly significant positive relationship between residual female remating interval and the residual number of progeny produced prior to remating (figure 2), with residuals generated following regression of each variable on female size (F ranged from 16.42 to 455.74, r^2 ranged from 0.307 to 0.925 and p ranged from less than 0.0002 to less than 0.0001). Monogamy-line females produced more progeny prior to remating than control-line females (figure 3; mean $\pm$ s.e.m. progeny for replicate A, control 99.0 ± 7.9; monogamy 129.1 ± 7.5; and for replicate B,

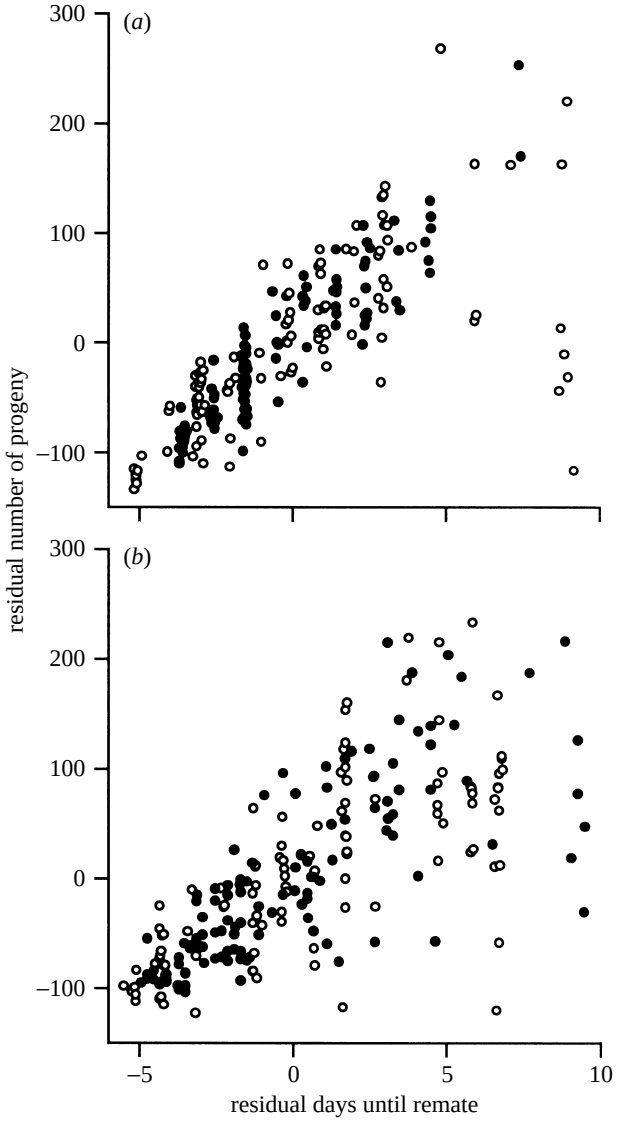

Figure 2. Relationship between residual number of progeny produced prior to remating and residual remating interval, after removing female body-size effects from both variables (slope $= 16.12$). (*a*) Replicate A and (*b*) replicate B. Solid circles, control-line females; open circles, monogamy-line females.

control 101.2 ± 7.5; monogamy 122.0 ± 8.0). This selection effect was highly significant in replicate A and there was a strong but non-significant trend in replicate B (table 3). Male mating order also explained a highly significant amount of this variation, but only in replicate B (table 3).

4. DISCUSSION

Evolutionary change in female remating behaviour following experimental removal of sexual selection provides insight into the selective pressures defining female polyandry in *D. melanogaster* under laboratory conditions. The two opposing predictions of the sexually antagonistic coevolution hypothesis are contingent upon the evolution of different forms of reduced male manipulation of females by monogamy-line males. It is thus necessary to consider the expression of male traits when interpreting the observed divergence in female remating behaviour. If males evolved reduced courtship manipulation of females

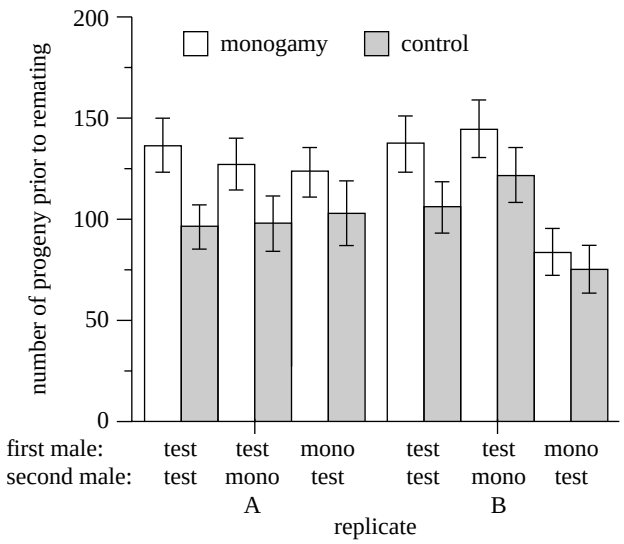

Figure 3. Number of progeny produced prior to remating with a second male by monogamy- and control-line females when presented with three different combinations of males. Bars indicate one s.e.m.

and females consequently evolved lowered resistance to courtship manipulation, then the monogamy-line females should have remated more quickly than the control-line females when courted by test males. If males evolved reduced ejaculate manipulation of females and females consequently evolved lowered resistance to ejaculate manipulation, then the monogamy-line females should have remated less quickly than the control-line females following insemination by test males.

Comparisons made within each male-order treatment clearly indicate that monogamy-line females were consistently slower to remate than control-line females (figure 1). Moreover, no significant 'second male' order effects were observed (table 2). These data clearly refute the hypothesis that the evolutionary change in female remating behaviour in these lines was due to sexually antagonistic coevolution of courtship-signal and receiver traits. This result is incongruous with the findings of Holland & Rice (1999) that male courtship rate diminished in the monogamy lines after 45 generations of selection. Holland & Rice's assay was to quantify the proportion of vials in each treatment for which courtship was observed in each of 12 instantaneous scans distributed throughout one day. Our results suggest that this parameter is not relevant to female remating behaviour. It may be that the quality of male courtship, rather than its frequency, is the major determinant of the probability of female remating, and this trait may not have diverged between selection lines. Alternatively, the treatment differences observed by Holland & Rice may have disappeared during the intervening 39 generations between the studies.

The observed pattern of monogamy-line females taking longer to remate than control-line females does support the sexually antagonistic coevolution hypothesis for ejaculate manipulation and female resistance. Moreover, conflict over the remating interval between females and their previous mates was empirically supported by the strong positive relationship between female remating

Table 3. *Results of analysis of covariance (ANCOVA) of the number of progeny produced prior to remating for each paired replicate*

variable	*F*	d.f.	*p*
replicate A			
monogamy versus control female	10.68	1,225	0.001
male mating order	0.21	2,225	0.81
female–male interaction	0.08	2,225	0.92
replicate B			
monogamy versus control female	3.28	1,230	0.07
male mating order	9.66	2,230	< 0.0001
female–male interaction	0.36	2,230	0.79

interval and total progeny production prior to remating (figure 2). Also, the significant male-order effect on female remating observed in replicate B is clearly attributable to 'first male' (i.e. ejaculate) effects (table 2). Specifically, monogamy-line and control-line females in replicate B remated more rapidly when initially mated to monogamy-line males than when initially mated to test males, irrespective of second males (figure 1). Although monogamy-line males are smaller than control-line males (S. Pitnick, unpublished data), the size of copulating males has been demonstrated to have no influence on the subsequent remating behaviour of their mates (Pitnick 1991).

It is important to consider, however, that any evolutionary reduction in female resistance to male manipulation following the removal of sexual selection is dependent upon the reduction of male manipulation (Holland & Rice 1999). The conclusions drawn from our data are therefore dependent upon one's interpretation of the post-hoc analysis of the propensity of females to remate during the first two days of the experiment. If one is inclined to place greater value on the entire data set then reduced ejaculatory manipulation evolved only within the replicate-B lines (table 2). Given that similar female effects were observed in both replicates, therefore, sexually antagonistic coevolution between male ejaculate and female remating traits is not a sufficient explanation for the divergence in female behaviour observed between the selection lines and alternative explanations must be considered. This interpretation is supported by results from another experiment that examined the competitive fertilization success of these monogamy- and control-line males after 81 generations of selection. Males were mated to females from a population unrelated to the selection lines, and no significant ejaculate or courtship effects on female remating were detected (S. Pitnick, unpublished data). On the other hand, if one accepts the post-hoc analysis, then reduced ejaculatory manipulation of monogamy-line females evolved in both replicates and the consistent divergence in female remating behaviour may be explained by the evolution of lowered female resistance to male ejaculatory manipulation.

Sexual-conflict theory has also been used to suggest that females of some species remate because the costs associated with refusing to copulate with ardent or persistent males may exceed the costs of remating (e.g. Arnqvist 1989; Watson 1993; Watson *et al.* 1998). Such female behaviour, termed 'convenience polyandry'

(Thornhill & Alcock 1983), is adaptive for females because they are 'making the best of a bad job'. Given that monogamy-line females, being confined with only one instead of three males, will receive less male harassment and thus suffer lowered costs of resisting copulation and, also, monogamy-line males are under selection to maximize their sole mates reproductive success, and therefore harmful coercion will be selected against, this hypothesis does predict the observed increase in the remating interval in monogamy-line females. This hypothesis, however, may have limited applicability to *D. melanogaster*. Frequency of female remating does not increase with increasing population density (Gromko & Gerhart 1984; Harshman *et al*. 1988), suggesting that the divergence in the remating interval between our lines is attributable to causes other than reduced male harassment. In addition, females of this species employ a variety of effective behaviours to thwart the sexual advances of undesired males, including decamping, kicking, wing flicking and ovipositor extrusion (Spieth 1952). Moreover, female cooperation, indicated by a stereotypical wing-spread behaviour, is required for males to mount and initiate copulation (Markow & Hanson 1981; Tompkins *et al*. 1982) under all but exceptional circumstances (Markow 2000).

An alternative, non-adaptive hypothesis that could explain our results is that there exists a common genetic control of remating in the two sexes. Thus, the observed divergence in female remating frequency would be a correlated response to changes in male mating frequency (Halliday & Arnold 1987). This hypothesis has been criticized on theoretical grounds (Sherman & Westneat 1988; but see Arnold & Halliday 1988). In addition, it was not supported by a comparative analysis of *Drosophila* (Schwartz & Boake 1992) or by empirical results from artificial selection experiments on *D. melanogaster* (Gromko & Newport 1988). Selection on females for increased or decreased remating speed generated significant responses whereas selection on males did not, indicating the absence of a genetic correlation between the sexes for variation in this trait (Gromko & Newport 1988). We did not assay the mating frequency of the selection-line males and so we cannot rule out the 'lack of sex limitation' hypothesis (Halliday & Arnold 1987) as a possible explanation of our results.

Alternatively, it may be that female remating behaviour in *D. melanogaster* has evolved in response to benefits accrued from remating. For example, in species where males transfer relatively few sperm (e.g. Pitnick 1993) or where females are not capable of prolonged sperm storage, females may remate to replenish their sperm supply (Gromko *et al*. 1984; Gromko & Markow 1993). The magnitude of this benefit is the same for females remating with the same male as for females remating with different males, so this hypothesis predicts no evolutionary change in remating behaviour as a consequence of monogamy selection, contingent upon there being no evolutionary change in the number of sperm delivered by males. Monogamous males evolve relatively smaller testes that produce significantly fewer sperm (S. Pitnick, unpublished data). The sperm-replenishment hypothesis might, therefore, predict that monogamy-line females would evolve to remate more frequently than control-line females. The opposite pattern was observed (figure 1), suggesting that other selective benefits influence the female remating interval more than the need to replenish sperm in *D. melanogaster*.

Numerous other direct and genetic benefits accrued by females from mating with multiple males have been proposed to explain remating by females (Walker 1980; Thornhill & Alcock 1983; Halliday & Arnold 1987; Keller & Reeve 1995; Jennions & Petrie 2000) and a recent meta-analysis of 122 experimental studies addressing the direct effects of multiple mating on female fitness in insects revealed that females generally benefit from multiple mating in terms of increased lifetime offspring production (Arnqvist & Nilsson 2000). Because monogamous mating eliminates all opportunities for females to obtain benefits from mating with multiple males, all of these hypotheses predict the observed decrease in remating frequency by monogamy-line females relative to control-line females.

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