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Males' evolutionary responses to experimental removal of sexual selection

Scott Pitnick[1]*, Gary T. Miller[1], Jennifer Reagan[1] and Brett Holland[2]

[1]*Department of Biology, Syracuse University, 108 College Place, Syracuse, NY 13244-1270, USA*
[2]*Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA*

We evaluated the influence of pre- and post-copulatory sexual selection upon male reproductive traits in a naturally promiscuous species, *Drosophila melanogaster*. Sexual selection was removed in two replicate populations through enforced monogamous mating with random mate assignment or retained in polyandrous controls. Monogamous mating eliminates all opportunities for mate competition, mate discrimination, sperm competition, cryptic female choice and, hence, sexual conflict. Levels of divergence between lines in sperm production and male fitness traits were quantified after 38–81 generations of selection. Three *a priori* predictions were tested: (i) male investment in spermatogenesis will be lower in monogamy-line males due to the absence of sperm competition selection, (ii) due to the evolution of increased male benevolence, the fitness of females paired with monogamy-line males will be higher than that of females paired with control-line males, and (iii) monogamy-line males will exhibit decreased competitive reproductive success relative to control-line males. The first two predictions were supported, whereas the third prediction was not. Monogamy males evolved a smaller body size and the size of their testes and the number of sperm within the testes were disproportionately further reduced. In contrast, the fitness of monogamous males (and their mates) was greater when reproducing in a non-competitive context: females mated once with monogamous males produced offspring at a faster rate and produced a greater total number of surviving progeny than did females mated to control males. The results indicate that sexual selection favours the production of increased numbers of sperm in *D. melanogaster* and that sexual selection favours some male traits conferring a direct cost to the fecundity of females.

Keywords: sexual selection; sexual conflict; sperm competition; body size; *Drosophila*

1. INTRODUCTION

In those rare species with strict monogamy, the reproductive interests of males and females are confluent. In all other species, conflict between the sexes is likely to arise. Conflict over the decision of whether or not to mate ('mating conflict') is widespread (Arnqvist 1997) because males tend to have higher potential reproductive rates and, thus, are much more ardent than females (Clutton-Brock & Parker 1992). However, between mates the majority of conflict is the result of post-copulatory sexual selection (i.e. sperm competition and cryptic female choice; Stockley 1997). Widely recognized as a potent force responsible for shaping behaviour, physiology and anatomy (Parker 1970; Eberhard 1996; Birkhead & Møller 1998), post-copulatory sexual selection may generate sexual conflict through three discrete processes. First, there may be conflict over how many gametes are dedicated to each mate. Such 'monopolization conflict' includes paternity costs to males of female remating (Parker 1970; Westneat *et al*. 1990) and fertility costs to females of males partitioning their sperm between successive mates (Warner *et al*. 1995). This form of conflict will generate selection for traits, such as copulatory plugs (Polak *et al*. 1998), anti-aphrodisiacs (Andersson *et al*. 2000) and mate guarding by males (Birkhead & Møller 1992), which enhance gamete monopolization through direct intervention of mate behaviour.

*Author for correspondence (sspitnic@ syr.edu).

Second, conflict may arise through physiological trade-offs between traits contributing to reproductive success. Such 'allocation conflict' is common because one sex (typically females) invests predominantly in offspring while the other sex invests predominantly in fertilization opportunities (Bateman 1948; Williams 1966; Trivers 1972; Parker 1979). For example, females will suffer a cost when males compromise their level of parental investment in favour of seeking matings with additional females.

The third kind of sexual conflict occurs when traits that are adaptive for one sex in reproductive competition have incidental negative effects on the opposite sex. The most notorious example of such 'by-product conflict' is the toxicity of male seminal-fluid proteins in *Drosophila melanogaster* (Fowler & Partridge 1989; Chapman *et al*. 1995) and *Caenorhabditis elegans* (Gems & Riddle 1996). Certain unidentified seminal proteins are known to increase the risk of female mortality. Because there may be no selective advantage in reducing mate longevity (but see Johnstone & Keller 2000), the harm to females is believed to be an incidental by-product of the beneficial aspects of these proteins for males: they mediate sperm competition (Harshman & Prout 1994; Clark *et al*. 1995; Civetta & Clark 2000).

The causes of monopolization conflict (e.g. female remating) can be obvious. Sex-specific traits resulting from selection generated by this kind of conflict are often intuitively recognized and their adaptive significance may be determined experimentally with relative ease (e.g. mate guarding (Birkhead *et al*. 1989) and copulatory plugs (Dickinson & Rutowski 1989)). In contrast, without

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a detailed understanding of specific physiological trade-offs (Stearns 1992), identifying the sources of and evolutionary responses to allocation conflict can be difficult. When considering the contributions of post-copulatory sexual selection to sexual conflict, the fact that mechanisms underlying differential male fertilization success are unknown for most species further limits our ability to elucidate instances of allocation conflict. Recognition of by-product conflict is even less intuitive, and demonstrating its role in the evolution of sex-specific traits requires examination of harm in one sex while manipulating the putative causal trait in the opposite sex (Chapman *et al.* 1995).

Recently, novel insights into sexual conflict have come from studies that have experimentally manipulated sexual selection. First, by artificially preventing females from coevolving with males in a laboratory population of *D. melanogaster*, Rice (1996) demonstrated that net male fitness can increase at the expense of female survival. Second, Holland & Rice (1999) replaced the naturally promiscuous mating system of *D. melanogaster* with enforced monogamy and random assignment of mates in replicate populations and, thus, eliminated any opportunity for pre- and post-copulatory sexual selection. They found that males of monogamous lines evolved to be less harmful to their mates, and monogamous populations evolved a greater net reproductive rate than their polyandrous controls. These studies suggested that experimental manipulation of sexual selection provides a valuable approach for detailed investigation of sex-specific traits arising through allocation and by-product conflict.

This study extended the work of Holland & Rice (1999) by examining evolutionary responses in male traits (i.e. body mass, testis mass, the number of sperm produced, sperm length and competitiveness in sperm competition) and the fitness consequences of trait divergence for both sexes in monogamy and paired control populations following 38–81 generations of selection. Three *a priori* predictions were tested: (i) male investment in spermatogenesis will be lower in monogamy-line males due to the absence of sperm competition selection, (ii) due to the evolution of increased male benevolence, the fitness of females paired with monogamy-line males will be higher than that of females paired with control-line males, and (iii) monogamy-line males will exhibit decreased competitive reproductive success relative to control-line males.

2. MATERIAL AND METHODS

The selection lines examined in the present study were the same as those reported on by Holland & Rice (1999) (see Holland & Rice's (1999) paper for details of the protocol by which the 'monogamy' and 'control' lines were established and maintained). In brief, two replicate (A and B) pairs of lines were established from a single ancestral wild-type population of *D. melanogaster* with each replicate consisting of a monogamous and 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. Selection continued for 81 generations, with traits measured at generations as indicated below.

All traits were measured in flies reared under standard conditions by transferring 150 eggs for each line to each of three 8-dr shell vials containing 8 ml of medium. On the day of eclosion, virgin males were collected following anaesthetization with CO_2. All data were collected blind; the vials were colour coded so that the investigators measuring the traits were not aware of line identity until data collection was completed. Male and female size was determined in all experiments by measuring the length of the thorax.

(a) *Sperm production*

In order to examine the effects of monogamy selection on sperm production, the dry masses of the body and testes were quantified for all lines following 61 generations of selection, sperm length was quantified after both 61 and 81 generations and the number of sperm cysts developing within the testes was determined after 81 generations.

(i) *Testis mass*

Dry testis and body masses were determined for each six- to nine-day-old male (equal age distribution among lines) ($n = 50$ males per line) by dissecting both testes into distilled H_2O following anaesthetization. Testes were transferred to a pre-weighed piece of aluminium foil and all remaining tissue was placed on another pre-weighed piece of foil. The samples were then dried at 60 °C for 24 h prior to weighing to the nearest 1.0 μg on a Cahn C-35 microbalance (Analytical technology, Inc., Boston, MA, USA).

(ii) *Sperm numbers*

One randomly chosen testis was dissected from each anaesthetized male using a technique that releases all sperm bundles intact from the testis without disrupting their relative position. As an index of sperm production rate (Pitnick & Markow 1994; Pitnick 1996), the number of sperm bundles present in a mid-testis cross-section was counted for each six- to eight-day-old male (equal age distribution among lines) ($n = 15$ males per line).

(iii) *Sperm length*

One seminal vesicle from each male was dissected into phosphate-buffered saline (PBS) on a subbed microscope slide and then ruptured with a fine probe. After more than 100 sperm were loose in saline, the preparation was dried in a 60 °C oven, fixed in methanol:acetic acid (3:1) for 1 min, rinsed in PBS and then mounted under coverslips in a mounting medium of glycerol and PBS (80/20 v/v). Digitized images of sperm were captured at a magnification of × 200 by a Dage CCD72 camera (Dage-MTI, Inc., Michigan City, IN, USA) mounted on an Olympus BX60 microscope (Olympus America, Inc., Melville, NY, USA). The lengths of five randomly chosen sperm were measured using NIH Image software (developed at the US National Institutes of Health) in order to determine the mean sperm length for each male ($n = 20$ males per line from generation 61 and $n = 15$ males per line from generation 81).

(b) *Male fitness*

Three separate experiments quantified the fitness consequences of changes in the sperm production characters described above and those of other variables that have responded to monogamy selection (Holland & Rice 1999). In the first experiment, which was performed after 38 generations of selection, male net competitive reproductive success was measured under conditions identical to those under which the control selection lines were maintained.

Next, single mating productivity was quantified after 66 generations of selection. The final experiment, which was performed after 81 generations of selection, quantified male ability to induce female refractoriness to subsequent courtship, male ability to coerce mating by non-virgin females and male sperm competitiveness, both when males were the first (i.e. P_1) and when they were the second of two successive mates (i.e. P_2) of females.

(i) *Net competitive reproductive success*

The competitive reproductive success of males was assayed under conditions identical to those encountered by control males throughout the selection process. On their day of eclosion, each virgin test male ($n = 190$ males per line) was placed in an interaction vial with two brown, dominant males (bw^D) and one wild-type female. After five days, all flies were transferred without anaesthesia to fresh culture vials, where they remained for 24 h before being discarded. All progeny eclosing from these single-day culture vials ($n = 659$ for all treatments combined) were scored for paternity in order to determine the proportion of progeny sired by the wild-type (i.e. selection-line) male. Vials were removed from the experiment if any of the adults were missing from or found dead within the mating or culture vials ($n = 36$ out of 760) or if no progeny were produced ($n = 65$ out of 760, distributed as $n = 11$, 20, 16 and 18 among the four lines M_A, C_A, M_B and C_B, respectively, where M represents monogamy and C represents control). These distributions were not significantly different (replicate A, $\chi^2 = 2.789$ and $p > 0.05$ and replicate B, $\chi^2 = 0.004$ and $p > 0.95$). Statistical analysis was conducted following angular transformation of the square root of the proportion of progeny sired data.

(ii) *Single-mating productivity*

Each ten-day-old virgin test male ($n = 40$ males per line) was aspirated along with a single five-day-old virgin sepia-eye (*se*/*se*) female into an 8-dr shell vial with medium and live yeast. All males were observed to mate. Males were aspirated from the vials within 1 h of copulation ending and measured prior to being discarded. Each female was transferred to a fresh vial on days 2, 3, 5, 9, 13 and 17. All females were measured on day 22, by which time no females were laying fertile eggs. All progeny eclosing from all vials were counted (total of 15 952 flies). Females producing no progeny ($n = 11$, distributed as $n = 2$, 5, 1 and 3 among the four lines M_A, C_A, M_B and C_B, respectively) and those dying in the first 12 days of the experiment ($n = 7$) were excluded from statistical analyses. Although the distributions of females producing no progeny were not significantly different (replicate A, $\chi^2 = 0.626$ and $p > 0.25$ and replicate B, $\chi^2 = 0.263$ and $p > 0.50$), their exclusion from statistical analysis was conservative, as more females mated to control-line males produced zero progeny than did females mated to monogamy-line males (see below).

(iii) *Sperm competitiveness and female remating*

Each male was mated with an *se*/*se* female who was also mated with a randomly assigned *se*/*se* male. Both mating orders were tested, with the order (*se*/*se*, wild) considered as a test of the 'offence' ability of the selection-line male to take precedence over sperm residing within the female. The other order (wild, *se*/*se*) was considered as a test of 'defense', determining how well the selection-line male's sperm resisted displacement or preemption by the *se*/*se* male's sperm.

In order to obtain initial matings, four- to five-day-old virgin *se*/*se* females were randomly assigned to treatments ($n = 50$ females per mating order per line) and each was paired with a single, four- to five-day-old virgin male within an 8-dr 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 allowed 2-h opportunities for remating on each successive day by aspirating two five- to ten-day-old virgin males of the appropriate genotype into their vial. After 2 h, males were removed from the vials of females that did not remate. This process continued for nine days, by which time 98% of females had remated. Those that did not remate ($n = 7$) were excluded from analyses and all remating females were combined in a single analysis. Females were provided with fresh vials every other day during the remating interval. All vials were retained in order to quantify the number of progeny produced prior to remating.

Whenever a female permitted a second male to mount her, the non-mating male was gently aspirated out of the vial and discarded. Following copulation, the mating male was removed, measured and discarded. The female was immediately transferred to a new vial with medium and live yeast and then transferred again after 24 h for each of the next two days. After day 3, females were measured and discarded. The daily transfer of females resulted in low-density rearing conditions for larvae (range = 26.7 ± 2.3–43.1 ± 3.5 for mean progeny per day for all treatments with no significant differences within days between treatments), thus ensuring that differential larval competitiveness was unlikely to confound our interpretation of sperm precedence patterns (Gilchrist & Partridge 1997). Once all progeny had eclosed from each female's three vials (mean ± s.e. = 112 ± 3 per female) ($n = 19\,748$ for all treatments combined), they were scored for genotype.

In order to ensure that cases where either the first or second matings were unsuccessful (i.e. no sperm transferred or the male was infertile) were excluded from the analyses, any females producing only first-male progeny following remating ($n = 5$, with females distributed as $n = 1$, 2, 2 and 0 among the four lines M_A, C_A, M_B and C_B, respectively) and those producing no progeny prior to remating and only second-male progeny after remating ($n = 7$, distributed as $n = 1$, 3, 2 and 1 among the four lines M_A, C_A, M_B and C_B, respectively) were removed from the study. In addition, females producing zero ($n = 11$, distributed as $n = 1$, 3, 5 and 2 among the four lines M_1, C_1, M_2 and C_2, respectively) or unusually few progeny (less than ten) following remating ($n = 10$, distributed as $n = 1$, 2, 2 and 5 among the four lines M_A, C_A, M_B and C_B, respectively) and those lost before the end of the experiment ($n = 5$) were excluded from statistical analyses. In total, 355 double matings distributed between eight treatment groups (four lines multiplied by two reciprocal mating orders) were studied.

Male success in sperm competition is typically measured as the proportion of total offspring produced that were sired by the first (P_1) or second (P_2) male following a second mating by a female. We calculated sperm precedence as $a/(b + 1)$, where a is the number of progeny sired by the selection-line male (wild-type) and b is the number of progeny sired by the *se*/*se* male (Hughes 1997), as this estimator of relative success is approximately unbiased (Haldane 1955). Thus, this ratio is a measure of P_1 in treatments where the wild-type male was first and of P_2 where the wild-type male was second. Cube-root transformations improved the fit of the ratios to a normal distribution and so were used for significance testing. Henceforth, we refer to these transformed ratios simply as the 'P_1 ratio' or 'P_2 ratio'.

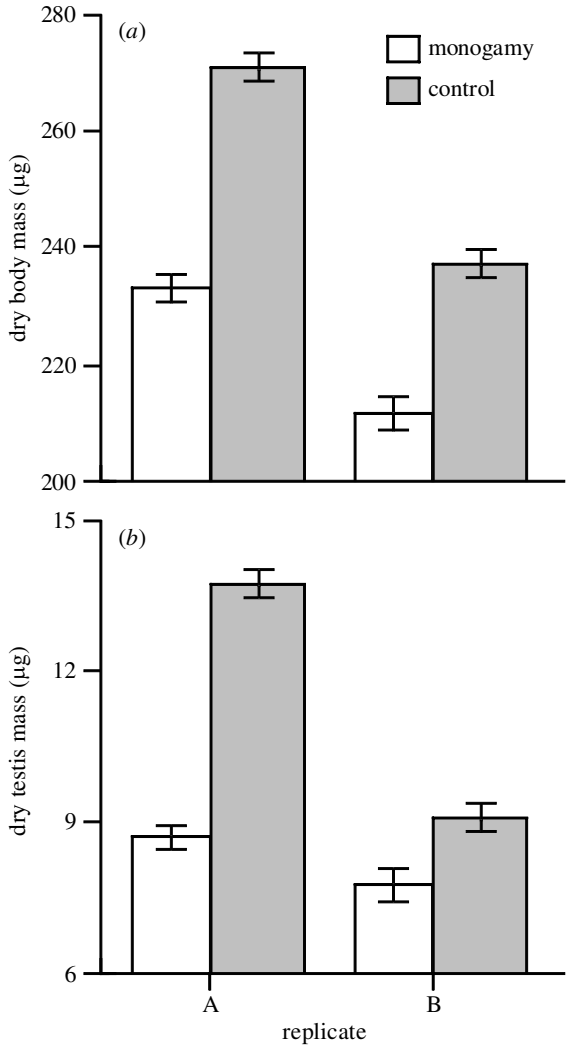

Figure 1. Mean dry mass of (*a*) all body tissue except the testes and (*b*) the testes. Bars indicate standard errors. ANOVA for selection effect: (*a*) $p < 0.0001$ and (*b*) $p < 0.0001$.

(c) *Statistical analyses and interpretation of responses to selection*

Whereas it can be argued that the selection lines are the experimental unit and, thus, that analyses should be based on an $n = 2$ basis per treatment, we believe that this approach is overly conservative and does not permit examination of the variation within lines or consideration of statistically significant responses that are not consistent between 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 be informative about character trade-offs and selection response mechanisms, we also recognize the limited potential for making strong inferences based on those traits and, thus, provide only limited discussion of such characters.

All variables were tested by a nested analysis of variance (ANOVA) with replicates (A and B) nested within selection regimes (monogamy and control). Because body size affects many of the traits examined, it was necessary to remove size effects statistically prior to the analysis of most variables. This was accomplished by generating residuals from regressions of traits on body size (i.e. thorax length or dry mass), with separate regressions run for each selection replicate. Although the data analyses were typically conducted on residual variation in traits following the removal of size effects, as specified below, all figures illustrate raw data for visual purposes only.

3. RESULTS

(a) *Body size*

Experimental manipulation of the intensity of sexual selection resulted in a significant divergence in body size, as measured by dry mass, with monogamy-line males smaller than control-line males (figure 1*a*) ($F_{1,2,196} = 144.56$ and $p < 0.0001$). There was also a significant replicate effect ($F_{1,2,196} = 57.05$ and $p < 0.0001$): replicate B flies were smaller than replicate A flies. Monogamy males were also significantly smaller than control males ($F_{1,2,707} = 581.30$ and $p < 0.0001$), as indicated by thorax length (using standard-reared males from all experiments) (first replicate: monogamy = 0.837 ± 0.003 mm and control = 0.889 ± 0.002 mm; second replicate: monogamy = 0.819 ± 0.002 mm and control = 0.870 ± 0.002 mm). Again, there was a significant replicate effect ($F_{1,2,707} = 36.96$ and $p < 0.0001$): replicate B flies were smaller than replicate A flies.

(b) *Sperm production*

Monogamy-line males had significantly lower residual testis mass than did control-line males (figure 1*b*) ($F_{1,2,196} = 25.99$ and $p < 0.0001$). There was also a significant replicate effect ($F_{1,2,196} = 4.792$ and $p < 0.01$), with greater divergence in replicate A than in replicate B flies. Monogamy-line males were also found to produce relatively fewer sperm than control-line males, as indicated by nested ANOVA of residual sperm cysts (figure 2*a*) ($F_{1,2,56} = 4.07$ and $p < 0.05$). There was no significant replicate effect ($F_{1,2,56} = 0.12$ and $p = 0.89$).

Sperm length was quantified after 61 generations of selection and then again after 81 generations. Significant relationships between sperm length and male thorax length were observed in both generation 61 and 81 assays in replicate B flies (linear regression, $p < 0.05$), but not in replicate A flies ($p > 0.28$). Nevertheless, all data were size corrected prior to analysis. Examination of figure 2*b* reveals that the sperm of monogamy-line males were longer than those of control-line males in both assays for replicate B flies, but not for replicate A flies. Due to the inconsistent evolutionary response in sperm length to alteration of the intensity of sexual selection, the nested ANOVAs of residual sperm length were statistically non-significant in both generation 61 ($F_{1,2,76} = 0.63$ and $p = 0.43$) and generation 81 ($F_{1,2,76} = 1.42$ and $p = 0.24$).

(c) *Male fitness*

The rate of progeny production of standard *se*/*se* females was consistently greater when paired with a monogamy-line male than with a control-line male ($F_{1,2,138} = 6.73$ and $p = 0.0105$ for total productivity). The relationship between female productivity and male thorax length was non-significant in all groups (linear regressions, $p > 0.26$).

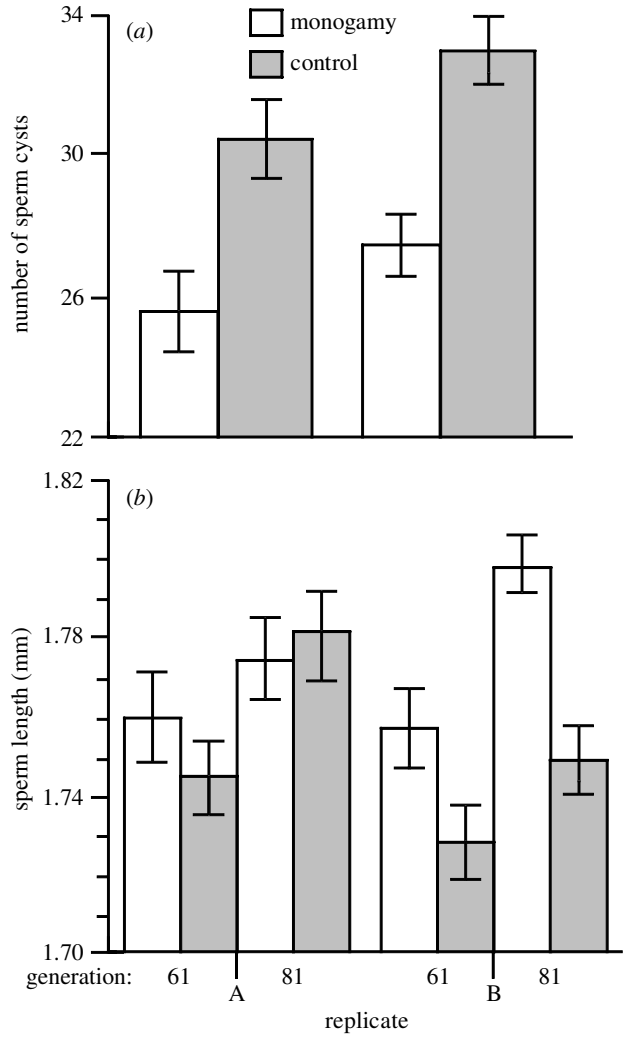

Figure 2. (*a*) Number of developing sperm cysts located at a mid-testis cross-section within a single testis and (*b*) mean sperm length. Bars indicate standard errors. ANOVA for selection effect: (*a*) $p < 0.05$ and (*b*) not significant.

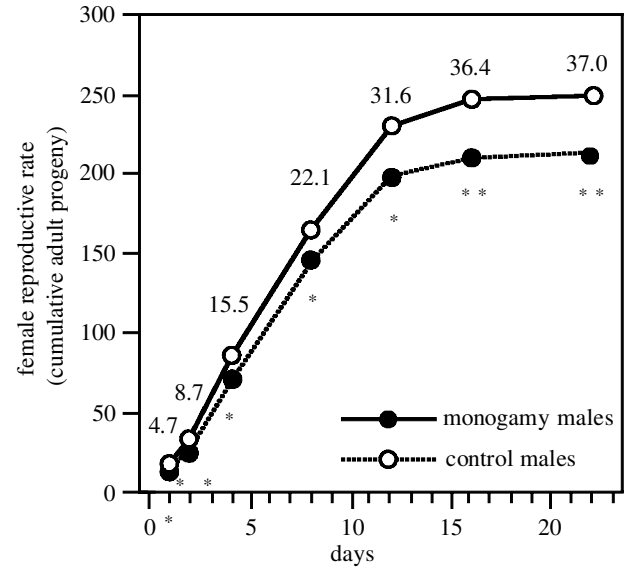

Figure 3. Cumulative number of mature (eclosed) progeny produced by surviving standard females following a single insemination. Numbers above curves indicate the difference between selection regimes in the mean number of progeny cumulatively produced by each measurement day. $^{*}p < 0.02$, $^{**}p < 0.01$.

Because the effect of replicate was non-significant ($F_{1,2,138} = 0.31$ and $p = 0.74$), data from the replicate lines were combined for illustration and analysis of cumulative progeny produced over time (figure 3). Females inseminated by monogamy-line males produced significantly more progeny than did females inseminated by control-line males on the first day of oviposition ($F_{1,140} = 6.07$ and $p < 0.02$), and the numbers of cumulative progeny produced remained significantly different throughout the experiment (figure 3).

Contrary to prediction, there was little to no difference between lines in the assays of competitive reproductive success. Monogamy-line males performed significantly less well than control-line males in the net competitive reproductive success experiment (figure 4*a*) ($F_{1,2,676} = 4.48$ and $p = 0.035$), as determined by the proportion (transformed by taking the arcsine of the square root) of progeny sired by each selection male (monogamy or control) when facing competition from two brown, dominant males. However, we suggest caution when interpreting this result given the marginal significance level of the overall test, the highly significant replicate effect ($F_{1,2,676} = 5.00$ and $p < 0.01$) resulting from the strong divergence in replicate B and general lack of divergence in replicate A (figure 4*a*) and the fact that male size was not recorded to permit statistical control of this variable.

Altering the intensity of sexual selection had no significant evolutionary effect on the ability of males to inhibit remating by their mates ($F_{1,2,174} = 0.08$ and $p = 0.77$) or on their ability to make non-virgin females mate with them during the 2-h exposure period ($F_{1,2,178} = 0.36$ and $p = 0.55$). Male thorax length was unrelated to female remating interval in all comparisons for both replicates (linear regressions, $p > 0.27$) and, thus, not statistically controlled for. With respect to the number of progeny produced by females prior to remating, there was a significant positive relationship between this variable and male thorax length in one comparison (replicate A P_1 experiment, $p < 0.05$). We therefore removed male size effects when analysing this variable. Selection regime had no effect on the residual number of progeny produced by females prior to remating to *se*/*se* males in either the P_1 experiment ($F_{1,2,174} = 0.03$ and $p = 0.87$) or the P_2 experiment ($F_{1,2,177} = 0.33$ and $p = 0.57$).

When analysing the P_1 ratios (male sperm defence ability) and P_2 ratios (male sperm offence ability) statistically, we controlled for the number of progeny produced by females prior to remating since this variable showed consistently significant negative relationships with the P_1 ratio (linear regressions, replicate 1: $F = 4.78$ and $p < 0.05$ and replicate 2: $F = 16.77$ and $p < 0.0001$) and positive relationships with the P_2 ratio (replicate 1: $F = 6.22$ and $p < 0.02$ and replicate 2: $F = 9.50$ and $p < 0.01$). These relationships are not surprising given that the more progeny a female produces prior to remating, the fewer sperm there are remaining to compete with the subsequent male's ejaculate. Statistically removing the variation explained by female sperm use prior to

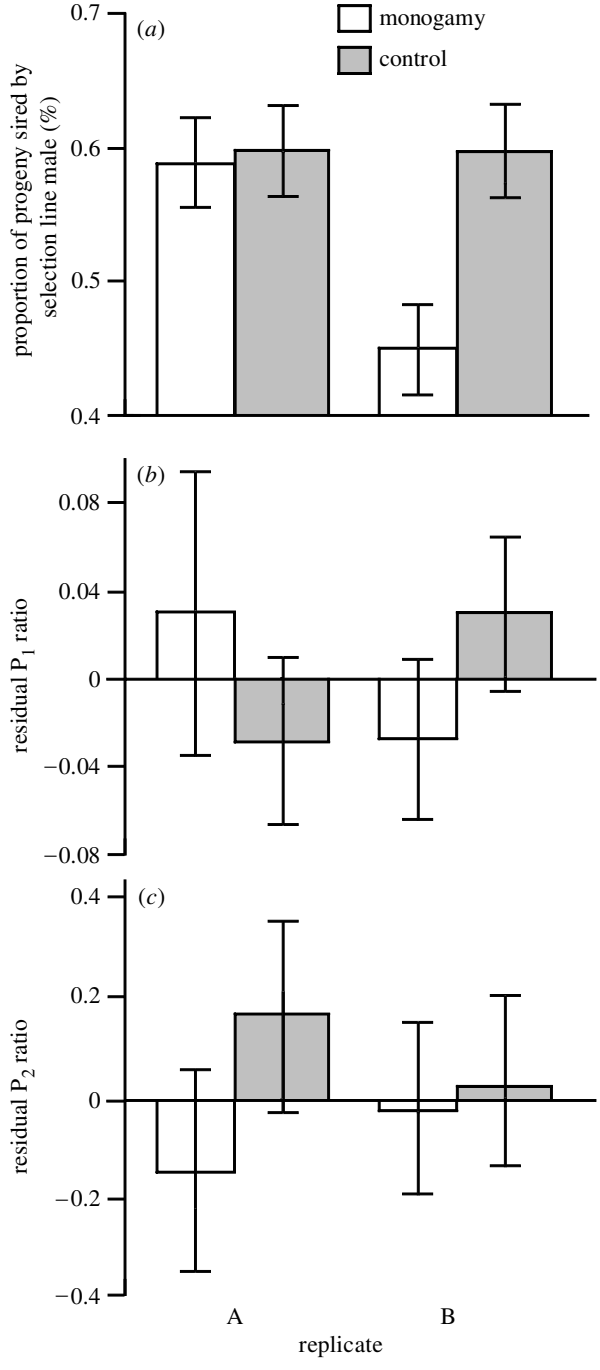

Figure 4. Competitive reproductive success of males. (*a*) Proportion of total progeny sired by the selection-line male when continuously paired with one *se*/*se* female and two *se*/*se* males. (*b*) Residual variation in the ratio of selection-line male progeny to *se*/*se* male progeny when the selection-line male mated first after controlling for the number of progeny produced prior to remating. (*c*) Residual variation in the ratio of selection-line male progeny to *se*/*se* male progeny when the selection-line male mated second after controlling for the number of progeny produced prior to remating and male size. See the text for details. Bars indicate standard errors. ANOVA for selection effect: (*a*) $p < 0.05$ and (*b*,*c*) not significant.

remating provides greater resolution for examination of differential male ejaculate competitiveness by 'evening the playing field' on which ejaculates compete. However, it should be noted that the analyses of the P_1 and P_2 ratios were also conducted without controlling for this variable and these delivered qualitatively similar outcomes. Male size was unrelated to the residual P_1 ratio in both replicates (linear regression, $p > 0.79$). However, male size showed a significant positive relationship with the residual P_2 ratio in the first replicate ($F = 6.49$ and $p < 0.02$) and, thus, was statistically controlled for when analysing the P_2 data. Nested ANOVAs revealed no significant effect of selection regime on either the residual P_1 ratios (figure 4*b*) ($F_{1,2,174} = 0.01$ and $p = 0.99$) or residual P_2 ratios (figure 4*c*) ($F_{1,2,177} = 0.99$ and $p = 0.32$). Replicate effects were not significant in either experiment ($p > 0.42$). Initial ANOVAs performed discretely on each selection replicate did generate a significant difference in the P_2 scores in replicate A (figure 4*c*) ($F_{1,87} = 8.94$ and $p < 0.01$). However, this difference became non-significant after controlling for male body size effects. The thorax lengths of females exhibited no statistically significant relationships with any measured variables in either the P_1 or P_2 analyses (linear regressions, $p > 0.05$).

4. DISCUSSION

Competition for mating between males is probably fierce in most *D. melanogaster* populations, with receptive females arriving at the breeding site being greeted by several courting males (Markow & Sawka 1992). The control lines, for which each female was paired with three males, therefore probably reflected a realistic level of pre-mating competition and a conservative level of post-mating competition, since repeated mating with the same male will be possible. We thus interpret divergence between lines as being primarily the result of evolutionary change within the monogamy lines. Nevertheless, because divergence in both the monogamy and control lines may have occurred, we discuss the responses by replicates (i.e. divergence between the monogamy and control lines) resulting from experimental manipulation of the intensity of sexual selection below. As mentioned above, we adopt a conservative stance with regard to interpretation of the significant divergence in traits that were not observed consistently among selection replicates. We did interpret consistent responses as being attributable to alteration in the intensity of sexual selection. However, it should be noted that alternative hypotheses of divergent responses arising through drift and/or inbreeding effects cannot be discriminated. Both inbreeding and drift were more likely to effect evolutionary change in the monogamy lines, given that the potential effective population size of nuclear genes in these populations was 50% lower than in the paired control lines due to the difference in the number of males. However, the differential inbreeding hypothesis, which could be argued to predict the evolution of smaller body size, relative testis mass and a reduction in the number of sperm produced within the monogamy lines, is not supported by the result that females mated to monogamy-line males achieve greater reproductive success than those mated to control-line males (figure 3).

One of the most striking responses to modifying sexual selection was a change in male body size (figure 1*a*). Another artificial selection experiment conducted with *D. melanogaster* (Promislow *et al.* 1998) similarly found divergence in male size following manipulation of sexual

selection. However, in that experiment females in all lines were permitted only a single insemination; what did differ between lines was the intensity of pre-copulatory sexual selection (one versus five males per female). Moreover, a positive correlation between male size and copulatory success has been demonstrated for *D. melanogaster* (Ewing 1961), whereas there is no statistically significant relationship between male size and success in sperm competition in this species (S. Pitnick, unpublished data). Thus, the observed size response in our lines may be primarily attributable to the removal of pre-copulatory rather than post-copulatory sexual selection.

Because the outcome of competition for fertilizing an egg will often be proportional to the representation of each male's sperm within the female (Parker 1970, 1990*a*; Simmons 1987), theory predicts an evolutionary increase in sperm production by males in lineages subjected to more intense sperm competition. This prediction has been widely supported through (i) comparative analyses of the correlation between sperm production and the intensity of sperm competition (Short 1979, 1981; Harcourt *et al*. 1981; Harvey & Harcourt 1984; Cartar 1985; Kenagy & Trombulak 1986; Møller 1988*a*,*b*, 1989, 1991; Ginsberg & Rubenstein 1990; Jennions & Passmore 1993; Bissoondath & Wiklund 1996; Kappeler 1997; Hosken 1997, 1998), (ii) comparison of sperm production between alternative male reproductive phenotypes when these types are associated with reproductive tactics that differ in exposure to sperm competition (Parker 1990*b*; Stockley *et al*. 1994; Gage *et al*. 1995; Taborsky 1998; Simmons *et al*. 1999), and (iii) investigations of facultative adjustment of the number of sperm produced or inseminated while varying the male's perceived risk of encountering sperm competition (Baker & Bellis 1989, 1993; Bellis *et al*. 1990; Gage 1991; Gage & Baker 1991; Gage & Barnard 1996; Wedell 1992; Simmons *et al*. 1993; Oppliger *et al*. 1998; Wedell & Cook 1999).

Our first prediction, that monogamy-line males would invest less in spermatogenesis, was supported, thereby providing experimental support for the predicted evolutionary response of sperm production to selection posed by sperm competition. Monogamy-line males were observed to have relatively smaller testes (figure 1*b*) with relatively fewer maturing sperm cysts than control-line males (figure 2*a*). These results indicate that sperm competition maintains the production of large numbers of sperm in *D. melanogaster*. Specifically, our results implicate the operation of numerical sperm competition, which occurs whenever the probability of a male's sperm fertilizing an egg is proportional to the representation of his sperm within the female (Parker 1970, 1982, 1993). This result is consistent with a recent study of the mechanisms underlying differential male fertilization success in *D. melanogaster* that suggested the degree of sperm displacement in this species is determined simply by the number of sperm transferred (Gilchrist & Partridge 2000). A recent study involving experimental removal of sexual selection in the dung fly *Scathophaga stercoraria* produced a similar reduction in relative testis size in monogamy-line relative to control-line flies (Hosken & Ward 2001; Hosken *et al*. 2001).

Because there is a trade-off between sperm size and the number of sperm produced (Pitnick 1996), sperm competition theory predicts that, when sperm competition is intense, males will produce the smallest sized sperm possible in order to maximize sperm numbers (Parker 1982; but see, for example, Gomendio & Roldan 1991; Briskie & Montgomerie 1992). A mathematical model has suggested that selection for males to provision offspring by increasing the size of their sperm will occur only in the complete absence of sperm competition (Parker 1982). In light of this model, the significant increase in sperm length in the second selection replicate is intriguing. However, given the inconsistent nature of the evolutionary response in sperm length, factors other than selection, including inbreeding and drift, represent viable interpretations for the result (Harshman & Hoffmann 2000).

The second prediction tested was that the fitness of females paired with monogamy-line males would be higher than that of females paired with control-line males. Experiments with *D. melanogaster* have demonstrated that male seminal fluid reduces female survival (Chapman *et al*. 1995; Rice 1996). This toxic effect of seminal proteins on females is thought to be an incidental by-product of the beneficial aspects of these proteins for males: they mediate sperm competition (Harshman & Prout 1994; Clarke *et al*. 1995; Civetta & Clarke 2000). A previous study of monogamy selection lines found that females inseminated by monogamy-line males lived significantly longer than females inseminated by control-line males, thus demonstrating that seminal fluid toxicity (or the quantity of the toxic component) is diminished when males evolve in the absence of sexual selection and supporting the conclusion that ejaculate toxicity is a sexually antagonistic trait (Holland & Rice 1999). Here, we examined the productivity of healthy females following a single insemination in order to explore whether such short-term effects of differential male benevolence on female reproductive success are discernible.

The consistently greater reproductive success of females inseminated by monogamy-line males over those mated with control-line males (figure 3) suggests that male benevolence towards females has evolved in a manner more immediate than that previously identified (i.e. reducing female longevity). The physiological/behavioural mechanism(s) underlying this effect are unknown. However, because the difference is evident on the first day of oviposition, when females are unlikely to be sperm limited, this effect is unlikely to be attributable to differences in the number of sperm transferred by males. Although sperm transfer was not quantified, the greater productivity of females mated with monogamy-line males was doubtfully a consequence of their having received more sperm, given that monogamy males produce fewer sperm (figure 2*a*). Finally, *D. melanogaster* males transfer many more sperm than females are capable of storing (Gilbert 1981) and egg laying is known to be influenced by seminal proteins rather than by variation in sperm supply (e.g. Chen *et al*. 1988; Kalb *et al*. 1993; Herndon & Wolfner 1995). It is also unlikely that this divergence is the result of differential larval survival brought about through sperm length differences. Given the consistent response in productivity between replicates, coupled with the inconsistent changes in sperm length, this explanation seems unlikely.

One plausible hypothesis for the mechanism underlying the divergence in single-mating productivity is that the seminal fluids of unselected flies additionally harm surviving females by lowering their fecundity. That is, females inseminated by monogamy-line males are able to produce more progeny and at a faster rate because they are injured less. This suggestion may at first seem incongruous with the knowledge that seminal fluids stimulate egg production and oviposition in *D. melanogaster* (Kalb *et al*. 1993; Herndon & Wolfner 1995). It is not known whether this stimulation is detrimental to female fitness (see discussion in Arnqvist & Nilsson 2000). We are suggesting that monogamy-line males have evolved a more benevolent form of chemical stimulation of oviposition by females, one that stimulates even greater oviposition by females than that normally observed. Alternatively, specific oviposition stimulants (e.g. Acp26Aa) (Herndon & Wolfner 1995) may not have diverged, but rather another ejaculatory product or other male effect that normally depresses oviposition may have evolved to be less harmful (or to be transferred in lesser quantity), thereby resulting in an increased net stimulation of oviposition in females. It is also possible that females make a greater investment in egg production, independent of any directed stimulation by males, when mating with monogamy-line males due to phenotypic traits not assayed in this study.

Our third *a priori* prediction, that monogamy-line males will exhibit decreased competitive reproductive success relative to control-line males, was generally not supported. Whereas a significant divergence in net competitive reproductive success was observed, this result was found despite an inconsistent response between selection replicates (figure 4*a*). Further, although a consistent trend of control-line males exhibiting superior sperm offensive ability relative to monogamy-line males was observed (figure 4*c*), no significant differences were found between lines in either the P_1 or P_2 experiments. Finally, there was no significant divergence in the ability of males to influence remating by females (but see Pitnick *et al*. 2001).

Lack of evolutionary divergence in measures of competitive male reproductive success is surprising considering the significant divergence in the number of sperm produced and in single-mating productivity. The development and maintenance of relatively large testes is energetically costly (see Pitnick 1996). Investment in relatively large testes is presumably maintained because the benefits of producing large numbers of sperm, which are accrued through enhanced competitiveness in sperm competition, outweigh the costs. Why then did monogamy-line males not perform less well in sperm competition? Similarly, whatever trait(s) confers enhanced female productivity presumably has pleiotropic costs in terms of male competitive reproductive success. Otherwise, all males would naturally express this trait and no divergence would arise in response to altering the intensity of sexual selection. It is of course possible that our assays were not sensitive to the proper trade-offs. For example, all males used in the sperm competition experiments were virgins aged several days beyond maturity. Consequently, all would have abundant sperm stores within their seminal vesicles. This protocol may have been insensitive to the costs of any reduction in the number of sperm produced that would perhaps be realized under more natural conditions of multiple mating by males. It is also worth noting that experimental removal of sexual selection in dung flies resulted in predicted divergence in both relative testis size and in competitiveness in sperm competition (Hosken *et al*. 2001).

In summary, experimental removal of sexual selection in *D. melanogaster* reveals that sexual selection favours larger males who invest a greater proportion of their total energy budget in sperm production. The greater reproductive success of females paired with monogamy-line males suggests that male and female reproductive interests are not naturally confluent in *D. melanogaster*. However, without a better understanding of the physiological mechanism underlying this selection response, it is not possible to identify the nature of the male trade-off responsible and whether it represents allocation conflict or by-product conflict between the sexes.

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