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# FEMALE RESISTANCE TO MALE HARM EVOLVES IN RESPONSE TO MANIPULATION OF SEXUAL CONFLICT

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*Abstract.*—The interests of males and females over reproduction rarely coincide and conflicts between the sexes over mate choice, mating frequency, reproductive investment, and parental care are common in many taxa. In *Drosophila melanogaster*, the optimum mating frequency is higher for males than it is for females. Furthermore, females that mate at high frequencies suffer significant mating costs due to the actions of male seminal fluid proteins. Sexual conflict is predicted to lead to sexually antagonistic coevolution, in which selection for adaptations that benefit males but harm females is balanced by counterselection in females to minimize the extent of male-induced harm. We tested the prediction that elevated sexual conflict should select for increased female resistance to male-induced harm and vice versa. We manipulated the intensity of sexual conflict by experimentally altering adult sex ratio. We created replicated lines of *D. melanogaster* in which the adult sex ratio was male biased (high conflict lines), equal (intermediate conflict lines), or female biased (low conflict lines). As predicted, females from high sexual conflict lines lived significantly longer in the presence of males than did females from low conflict lines. Our conclusion that the evolutionary response in females was to the level of male-induced harm is supported by the finding that there were no female longevity differences in the absence of males. Differences between males in female harming ability were not detected. This suggests that the response in females was to differences between selection treatments in mating frequency, and not to differences in male harmfulness.

*Key words.*—Accessory gland proteins, *Drosophila melanogaster*, experimental evolution, mating frequency, sex ratio, sexually antagonistic coevolution.

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There can be widespread conflicts of interest, both within and between the sexes, over mating and reproduction. For example, conflict is expected over access to matings (Darwin 1859, 1871; Parker 1979), choice of mate (Darwin 1871; Andersson 1982; Arnqvist 1992), mating frequency (Bateman 1948; Arnqvist and Rowe 1995), and the level of parental care (Trivers 1972; Clutton-Brock 1991). Parker first realized that traditional models of sexual selection do not explain situations in which adaptations in males decrease the fitness of their mates (Parker 1979). This type of sexual conflict is expected to lead to sexually antagonistic coevolution, in which adaptations that benefit males but harm females are selected in males and adaptations that minimize the extent of male harm are selected in females (Parker 1979; Rice 1996, 2000). Such conflict has the potential to generate rapid evolutionary change leading to reproductive isolation (Parker and Partridge 1998; Gavrilets 2000; Martin and Hosken 2003). Male harm to females may result from an unselected side effect of male-benefit adaptations (Parker 1979; Chapman et al. 1995; Morrow et al. 2003), or could instead increase male fitness directly (Crudgington and Siva-Jothy 2000; Johnstone and Keller 2000; C. R. Lessells, unpubl. data).

Evidence for sexually antagonistic coevolution comes from a variety of sources. Life-history studies combined with genetic manipulations have proved particularly powerful in the analysis of sexual conflict in *Drosophila melanogaster*. In this species there is a potential conflict over how often to mate. The optimum mating frequency is higher for males than it is for females (Bateman 1948). Furthermore, females that are courted at high rates (Partridge and Fowler 1990) and that mate at high frequencies (Fowler and Partridge 1989) suffer significant survival and lifetime reproductive costs. The cost of mating in females results from the effects of accessory gland proteins (Acps) transferred with sperm by mating males (Chapman et al. 1995). Accessory gland proteins have a variety of male-benefit functions. They are essential for normal sperm storage (Neubaum and Wolfner 1999) and success in sperm competition (Harshman and Prout 1994; Chapman et al. 2000). They also reduce female receptivity and increase egg-laying rate (Chen et al. 1988; Herndon and Wolfner 1995; Chapman et al. 2003a; Liu and Kubli 2003). The deleterious effects of Acps on females are thought to be a side effect of these male-benefit actions (Chapman et al. 1995; Morrow et al. 2003).

Comparative data have also been useful for detecting sexual conflict (Arnqvist and Rowe 2002a, b). Rapid evolutionary change in male reproductive genes (Begun et al. 2000; Swanson et al. 2001a) has also been taken as evidence of strong selection arising from sexually antagonistic coevolution. However, specific sexual conflict genes have not yet been identified and, furthermore, rapid evolution in reproductive genes is also predicted by sperm competition and cryptic female choice (Swanson et al. 2001b; Miller and Pitnick 2002).

Artificial selection has proved useful for detecting sexually antagonistic coevolution (Rice 1996; Holland and Rice 1999; Hosken et al. 2001; Martin and Hosken 2003). *Drosophila melanogaster* males allowed to adapt to a static female phenotype mated more often and sired more offspring than did control males, suggesting that they evolved greater fitness when released from the evolutionary constraints of counteradaptation in females (Rice 1996). Sexual conflict has also been investigated by imposing monogamy in polyandrous species. Under monogamy, differences in the genetic interests

of the sexes, and hence the intensity of sexual conflict, are minimized (Holland and Rice 1999; Hosken et al. 2001; Martin and Hosken 2003). *Drosophila melanogaster* females were found to survive longer following single matings to ''monogamy males'' compared to control, ''polyandry males'' (Holland and Rice 1999). Monogamy females also died faster than controls when continually housed with control males (Holland and Rice 1999). Therefore, monogamy males may have been less harmful to females, and monogamy females less resistant to male-induced harm. A potential problem with this (Holland and Rice 1999) and similar studies (Hosken et al. 2001) is that the effective population size of monogamy lines was smaller than that of control, polyandry lines. Thus, some findings, for example, reduced male competitiveness and reduced male harm to females under monogamy, are also predicted by the higher inbreeding and reduced body size in these lines (Sharp 1984; Pitnick et al. 2001; Snook 2001; Pitnick and García-González 2002; Chapman et al. 2003b). This makes the conclusions of these studies equivocal.

In this study we used a novel experimental design to test whether manipulating the level of sexual conflict leads to predictable changes in the level of female resistance to male-induced harm. We manipulated sexual conflict in *D. melanogaster* by varying the adult sex ratio, and produced three replicate lines each of male-biased (MB), equal-sex (ES) and female-biased (FB) sex-ratio treatments. In addition, all treatments permitted multiple mating by females, which is characteristic of natural populations (Harshman and Clark 1998; Imhof et al. 1998). All lines were derived from a laboratory-adapted, outbred wild-type population. We predicted that females from MB lines would be subject to frequent mating attempts from males and therefore should mate at a higher frequency than ES females who in turn should mate more than FB females. Thus, sexual conflict was predicted to be most intense in the MB lines, intermediate in the ES lines, and least intense in the FB lines. Females from the MB lines should therefore evolve the strongest resistance to male-induced harm, followed by females from the ES and then FB lines. We also expected males from the MB lines to evolve greater harmfulness to females than males from the ES followed by FB lines. We tested female resistance to male-induced harm by measuring the survival of females from the selection treatments when kept with wild-type males. We tested male harmfulness by measuring the survival of wild-type females housed continually with males from the selection treatments.

## Materials and Methods

### *Wild-Type Stock*

The Dahomey wild-type stock was collected in 1970 and has been maintained since then in four population cages with overlapping generations. Each Dahomey stock cage was supplied with three bottles (189 ml each) containing 70 ml of sugar-yeast (SY) food (100 g autolysed yeast powder, 100 g dextrose, 20 g agar, 30 ml nipagin (10% w/v solution), 3 ml propionic acid, 1 L water) every week. Bottles were removed after 28 days. All cultures were maintained at 25°C in non-humidified rooms on a 12:12 h light: dark cycle.

### *Manipulation of Sexual Conflict by Experimental Evolution*

To obtain flies for the selection treatments, eggs were collected from the four Dahomey cages by placing Petri dishes filled with a grape juice medium (50 g agar, 600 ml red grape juice, 42.5 ml nipagin (10% w/v solution), 1.1 L water) smeared with yeast paste inside each cage. First instar larvae were then picked from the dishes and placed in batches of 100 into glass vials (75 mm height × 25 mm diameter) containing 7 ml of SY food and live yeast. After all flies had eclosed, the adults were mixed, sexed under $CO_2$ anaesthesia, and randomly allocated to one of three selection treatments. Three replicate lines each of male biased (MB, 75 males and 25 females), equal sex (ES, 50 males and 50 females), and female biased (FB, 25 males and 75 females) sex ratio treatments were set up (i.e., nine lines in total). Each line was maintained in a plastic cage (220 × 140 × 85 mm) with a gauze-covered top. In each cage flies had access to water via two water-filled vials with cotton wool wicks. Flies were fed with two vials of SY food with added live yeast every two or three days. Live yeast was nonlimiting across all selection regimes, as indicated by excess yeast remaining in all vials each time the food was changed. Nine days after the cages were set up eggs were collected from each cage on grape plates smeared with yeast paste. The majority of eggs on the egg collection plates were allowed to hatch before larvae were collected, thus minimizing selection on early egg hatchability. Three hundred first instar larvae of each line were picked and raised at standard density (100 larvae per vial). Standard density culturing minimized competition between larvae. To minimize selection on development time, all adults were allowed to eclose over two days before being allocated to the same sex-ratio treatment and replicate number as their parents and maintained as described above. All subsequent generations were maintained using the same protocol.

### *Courtship and Mating Frequencies during Selection*

Courtship and mating frequencies within the selection cages were measured in a series of snapshot observations in generation 31. During the morning of each of six days throughout the selection period, 10 observations were made on each of the nine cages (with at least a 20-min gap between each observation to avoid counting matings twice). During each observation every cage was viewed from three sides in turn. This method minimized double counting and provided a good index of the level of courtship activity. The number of matings and courtship events (wing display directed at a female or attempted mounting) was recorded for each cage. Any dead flies were removed and counted, to ensure that correct sample sizes were used in calculations of courtship and mating frequencies. Courtship and remating frequencies observed were thus a representative sample of total courtship activity and total remating frequency.

### *Response to Selection in Females*

#### *Longevity, mating frequency, fecundity, and egg-to-adult viability of selection line females continually housed with wild-type males*

Female resistance to male-induced harm was tested after 18 generations of selection, by measuring the longevity of

selection females housed with wild-type males. Eggs from all selection line and wild-type Dahomey cages were collected on grape plates over a 16-h period. Dahomey eggs were placed at a density of 400 eggs per SY bottle, using a standard density culturing method (Clancy and Kennington 2001). Dahomey eggs were collected every seven days throughout the experiment to provide fresh, replacement males. Selection line eggs were allowed to hatch and 400 first instar larvae from each replicate of each selection treatment were placed into SY vials in batches of 100. One hundred females from each replicate of each selection treatment and 900 Dahomey males were collected within 8 h of eclosion to ensure virginity. The virgin flies were sorted into separate sexes under ice anaesthesia and placed in vials in single sex batches of 10. One bottle of Dahomey flies was allowed to emerge and was maintained in bottle culture with added live yeast, to provide spare males to replace any that died. All subsequent fly transfers were carried out using $CO_2$ anaesthesia, and all experimental flies were maintained in vials containing 7 ml SY food with added yeast.

One day after eclosion, selection females were split into batches of five and placed into fresh vials. Five Dahomey males were added to each of the vials already containing five selection females (20 vials, $n = 100$, for each replicate of each selection treatment). Flies were transferred onto fresh food every two or three days throughout the experiment and an equal sex ratio was maintained in each vial, using spare Dahomey males if necessary. As females died, vials were combined to maintain fly density at five females per vial. Dead flies were removed each time flies were transferred to new food and every seven days all males were discarded and replaced with fresh day-old Dahomey males. Female deaths were recorded six days a week until almost all (>98%) of the females had died.

The mating frequencies of experimental flies were recorded twice a week from 10 observations made on each vial at least 20 min apart. The fecundity of a sample of females from each replicate of each selection line was also measured twice a week. In each sample, 15 females were taken from each line and placed singly with one male each in a vial for 24 h, after which they were returned to their original groups. Eggs laid in the 24 h period were immediately counted and the vials retained in order to count progeny. The number of adult offspring divided by the number of eggs laid gave a score of egg-to-adult viability. For subsequent fecundity measures, a different set of 15 females was chosen from each line (returning to the first sample when all females within a line had been used). The use of different groups of flies for each fecundity measure minimized differential treatment of experimental females.

After 22 generations of selection, a replicate longevity experiment was performed, using double the sample size of females ($n = 200$ for each replicate of each selection treatment, method as described above).

### *Longevity of once-mated selection females in the absence of males*

To test whether longevity differences in females were attributable to resistance to male-induced harm, or instead reflected intrinsic differences in survival between the selection treatments, we measured the longevity of once-mated selection females, after 26 generations of selection. Egg collection, rearing of larvae, and adult virgin collection were as described above, except that Dahomey virgin males were placed in groups of 50 into yeasted SY bottles. One day after eclosion, selection females were placed in groups of 50 into the bottles containing 50 Dahomey males and were left for 48 h to allow all females to mate and initiate egg laying (Partridge and Fowler 1990). The flies were then anaesthetized and sorted into separate sexes. Selection females were placed in groups of 10 into yeasted SY vials and the males were discarded. Females were transferred into fresh, yeasted SY vials every two or three days for the duration of their life and dead flies were removed at each transfer. Deaths were recorded six days a week and vials were combined as females died to keep density at 10 females per vial whenever possible.

## *Response to Selection in Males*

### *Longevity, mating frequency, courtship frequency, fecundity, and egg-to-adult viability of wild-type females continually housed with selection males*

To measure the extent of male-induced harm to females, the longevity of wild-type females housed with selection males was tested after 33 generations of selection. Initial egg collection and larval rearing were as described above. The collection of selection line eggs was repeated every seven days throughout the experiment to provide fresh replacement selection males. The experiment was conducted as above, except that selection males and Dahomey females were used and courtship events (wing display directed at a female or attempted mounting) were recorded in addition to matings.

### *Body Size, Accessory Gland, and Testis Size of Selected Flies*

To document any morphological differences between the selection treatments, we measured the body size, accessory gland size, and testis size of flies reared at standard densities from generation 32 of selection. Approximately 50 flies of each sex from each replicate of each selection treatment were placed in yeasted SY bottles and allowed to mate for four days. The sexes were then separated and females were immediately frozen at −80°C. Males were maintained in groups of five in yeasted SY vials for 10–12 days to allow sufficient time for replenishment of the contents of testes and accessory glands (Bangham et al. 2002). Males were dissected as in Bangham et al. (2002) in phosphate-buffered saline on a glass slide, and images of the accessory glands and testis were captured from a compound microscope (100×) using a video camera attached to a Macintosh computer. The NIH Object Image program (vers. 1.62n3 by Norbert Vischer, available at http://simon.bio.uva.nl/object-image.html) captured all images and the polygon tool was used to measure the perimeter and area of testes and accessory glands. For body size, we used a measure of wing area (Gilchrist and Partridge 1999). The right-hand wings of males and females from all selection treatments were mounted on glass slides and were measured using the NIH Object Image program.

### *Data Analysis*

Except where stated, data analysis was performed using the software package JMP 5 (SAS Institute 2002). Normality and homogeneity of variances of all raw data and residuals from models were checked by Shapiro-Wilk (Shapiro and Wilk 1965) and Bartlett's tests (Zar 1999). Where required, data were transformed (log, inverse log, power or a combination of these) to homogenize variances and/or normalise data. The numbers of matings and courtship events were summed across observation days and compared using a one-way analysis of variance (ANOVA). When required, multiple comparisons were made using Student-Newman-Keuls (SNK) tests (Zar 1999). To determine accurate probabilities from q-values for SNK tests we used the software program ''R'' (Ihaka and Gentleman 1996). To compare the longevity of flies from different selection treatments, a Cox's proportional hazards regression (Cox 1972) was performed on survival data to provide unbiased estimates of survival risk for each line. Survival risk ratios were then compared across selection treatments using one-way ANOVA followed, where necessary, by SNK tests. Where longevity was assayed in two replicate experiments, probabilities across experiments were combined (Sokal and Rohlf 1995). We analyzed fecundity and egg-to-adult viability data with Kruskal-Wallis tests, excluding data from lines where the number of surviving females was ≤5. Critical *P*-values for fecundity and egg to adult viability analyses were corrected for multiple comparisons using the sequential Bonferroni method (Rice 1989). Where differences occurred, nested ANOVAs were performed. The two effects in the model were selection treatment (fixed effect) and replicate (random effect) nested within selection treatment (restricted maximum-likelihood method). Mean absolute body size, accessory gland, and testis sizes were compared between selection treatments using a one-way ANOVA. Allometry between testis and body size and accessory gland and body size was also compared across selection treatments, by comparing replicate means between treatments using a one-way ANOVA.

## RESULTS

### *Courtship and Mating Frequencies during Selection*

#### *Females*

The number of matings per female and the number of courtship bouts per female were highest in the MB, followed by ES, followed by FB lines, as expected. There were significant differences between selection treatments in the total number of matings per female over the six observation days (log transformed) (Fig. 1, $F_{2,6} = 8.25$, $P = 0.019$). Females in MB lines mated significantly more than females in FB lines ($\bar{X}$ and SE: MB = 0.67 ± 0.16 matings per female, FB = 0.23 ± 0.01 matings per female, $P = 0.018$) and ES lines ($\bar{X}$ and SE: ES = 0.30 ± 0.04 matings per female, $P = 0.027$). There were no significant differences in the numbers of matings between ES and FB lines ($P = 0.35$). There were significantly more total courtships per female (log transformed) observed in the MB compared to the ES followed by the FB lines ($F_{2,6} = 51.04$, $P = 0.0002$; $\bar{X}$ and SE: MB = 20.80 ± 3.04 courtship events per female, ES = 8.37 ± 0.80 courtship events per female, FB = 4.18 ± 0.18 courtship events per female; MB versus FB, $P = 0.0001$; MB versus ES, $P = 0.001$; ES versus FB, $P = 0.005$). These results indicate that the intensity of sexual conflict varied as anticipated during selection, being most intense in the MB lines, followed by the ES then FB lines.

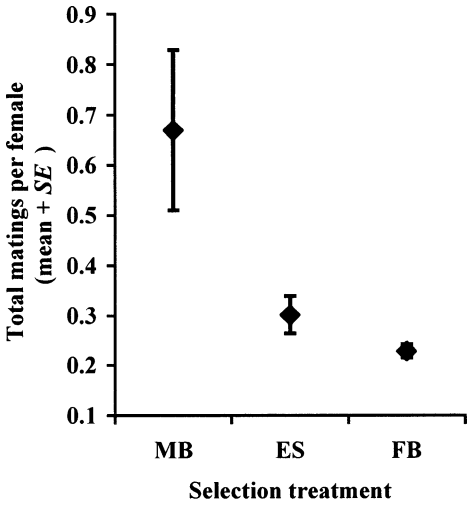


FIG. 1. The mean (± SE) total number of matings observed per female during selection. Matings were observed on six days of the 10-day selection period. Ten observations were made on each cage on each day, with at least a 20-min gap between each to avoid double counting matings.

#### *Males*

As expected from above, the number of matings per male and the number of courtship bouts per male were highest in the FB, followed by ES, followed by MB lines. There were significant differences between selection treatments in the total number of matings per male over the 6 observation days ($F_{2,6} = 31.77$, $P = 0.0006$). Males in MB lines mated significantly more often than males in FB lines ($\bar{X}$ and SE: MB = 0.22 ± 0.05, FB = 0.69 ± 0.04, $P = 0.0007$) but not significantly more than ES lines ($\bar{X}$ and SE: ES = 0.30 ± 0.04, $P = 0.26$) and males in ES lines mated significantly more often than males in FB lines ($P = 0.0008$). There were significant differences in the number of courtship events per male ($F_{2,6} = 13.41$, $P = 0.006$). The number of courtship events per male differed between MB and FB lines ($\bar{X}$ and SE: MB = 6.98 ± 0.99, FB = 12.73 ± 0.50, $P = 0.006$) and ES and FB lines ($\bar{X}$ and SE: ES = 8.43 ± 0.88 $P = 0.01$) but not between MB and ES lines ($P = 0.26$).

### *Response to Selection in Females*

#### *Longevity, mating frequency, fecundity, and egg-to-adult viability of selection line females continually housed with wild-type males*

There were significant, repeatable differences in female survival in the presence of wild-type males across both replicate survival experiments (Fig. 2, combined probabilities from both survival experiments, $\chi^2_4 = 12.97$, $P = 0.011$). MB and ES line females both had significantly higher survival

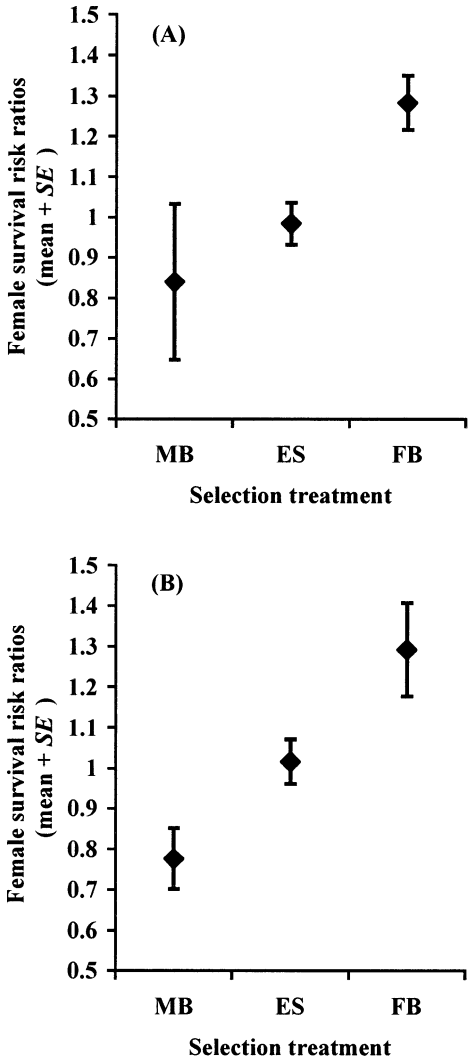


FIG. 2. Survival risk ratios (mean ± SE) of selection line females continually housed with wild-type males. Results of two replicated experiments performed at generations 18 (A) and 22 (B) are shown. Note that high risk ratio values indicate low survival.

(i.e., lower risk ratios) than FB line females (combined probabilities from both survival experiments, MB versus FB, $\chi^2_4 = 13.52$, $P = 0.009$; ES versus FB, $\chi^2_4 = 9.58$, $P = 0.048$). MB line females had higher, but not significantly higher, survival (i.e. lower risk ratios) than ES line females (combined probabilities from both survival experiments, $\chi^2_4 = 6.39$, $P = 0.17$). Mean survival values (days ± SE) for the first and second experiments respectively were: MB1, 28.25 ± 0.74, 27.26 ± 0.58; MB2, 24.49 ± 0.81, 25.03 ± 0.64; MB3, 31.07 ± 0.82, 27.59 ± 0.62; ES1, 26.80 ± 0.63, 25.54 ± 0.56; ES2, 27.09 ± 0.78, 24.05 ± 0.60; ES3, 26.35 ± 0.76, 24.93 ± 0.60; FB1, 24.10 ± 0.84, 24.27 ± 0.59; FB2, 24.28 ± 0.69, 22.73 ± 0.51; FB3, 24.68 ± 0.77, 24.01 ± 0.53. These differences in female survival could not be explained by differences in mating frequency between treatments (measured during the first replicate experiment at generation 18, $F_{2,6} = 0.24$, $P = 0.79$). The survival results provide evidence for a significant increase in resistance to male-induced harm in females from the MB relative to the FB treatments, with intermediate resistance in the ES females.

The life-history measurements from the first replicate experiment at generation 18 show that there were no significant differences in fecundity attributable to selection effects (Fig. 3A). Significant differences in fecundity on days 3 and 5 (Kruskal-Wallis tests; Day 3, $\chi^2_{8,} = 42.86$, $P < 0.0001$, critical $P = 0.0056$; Day 5, $\chi^2_8 = 25.03$, $P = 0.0015$, critical $P = 0.0063$) were attributable to replicate and not selection treatment differences (nested ANOVA model on square transformed data: day 3, selection effect, $F_{2,125} = 1.0426$, $P = 0.41$; day 5, selection effect, $F_{2,120} = 0.52$, $P = 0.62$). Day 3 median (lower and upper quartile) fecundity values were: MB1, 91.5 (87.75, 100.25); MB2, 68 (50, 74); MB3, 81 (61, 86); ES1, 91 (75, 99); ES2, 83 (66, 85); ES3, 98 (88, 107); FB1, 88 (82, 90); FB2, 92 (89, 99); FB3, 88 (73, 104). Day 5 median (+ lower and upper quartile) fecundity values were: MB1, 86 (80, 95); MB2, 84.5 (77, 94); MB3, 73.5 (65.5, 79.5); ES1, 90.5 (82.75, 100); ES2, 76 (59.5, 89.25); ES3, 96.5 (87.5, 111.5); FB1, 91 (59, 99); FB2, 89 (78, 99); FB3, 88.5 (75.5, 97.5). There was also no significant effect of selection on egg-to-adult viability (i.e., number of progeny/number of eggs, Fig. 3B). The one significant difference on day 5 (Kruskal-Wallis test, $\chi^2_{8,} = 23.24$, $P = 0.0031$, critical $P = 0.01$) was attributable to random variation between replicates and not to differences between selection treatments (nested ANOVA on day 5 egg-to-adult viability data twice inverse log transformed and raised to the power of 1.5; selection effect, $F_{2,119} = 2.14$, $P = 0.20$). Day 5 median (lower and upper quartile) egg-adult viability values were: MB1, 0.90 (0.77, 0.93); MB2, 0.72 (0.67, 0.81); MB3, 0.76 (0.70, 0.87); ES1, 0.82 (0.74, 0.91); ES2, 0.91 (0.85, 0.94); ES3, 0.88 (0.83, 0.92); FB1, 0.86 (0.73, 0.95); FB2, 0.90 (0.87, 0.95); FB3, 0.92 (0.62, 0.94). The results show that the longevity differences between females from the different selection treatments were not accompanied by detectable differences in mating frequency, age-specific fecundity or egg-to-adult viability.

### *Longevity of once-mated selection females in the absence of males*

There were no significant differences between selection treatments in the survival risk ratios of once-mated females in the absence of males (risk ratios, $\bar{X}$ and SE: MB = 0.99 ± 0.34, ES = 1.30 ± 0.29, FB = 0.92 ± 0.09; $F_{2,6} = 0.58$, $P = 0.59$). Mean survival values (days ± SE) were: MB1, 36.35 ± 0.80; MB2, 43.32 ± 1.29; MB3, 43.81 ± 1.37; ES1, 42.36 ± 0.97; ES2, 33.37 ± 1.07; ES3, 38.47 ± 1.34; FB1, 38.24 ± 1.29; FB2, 41.29 ± 0.93; FB3, 42.93 ± 1.19. This finding confirms that the differential longevity of selection females in the presence of males (Fig. 2) is a specific response to males and does not stem from differences between treatments in intrinsic female survival.

## *Response to Selection in Males*

### *Longevity, mating frequency, courtship frequency, fecundity, and egg-to-adult viability of wild-type females continually housed with selection line males*

There were no significant differences between the survival of wild-type females housed with males from the different

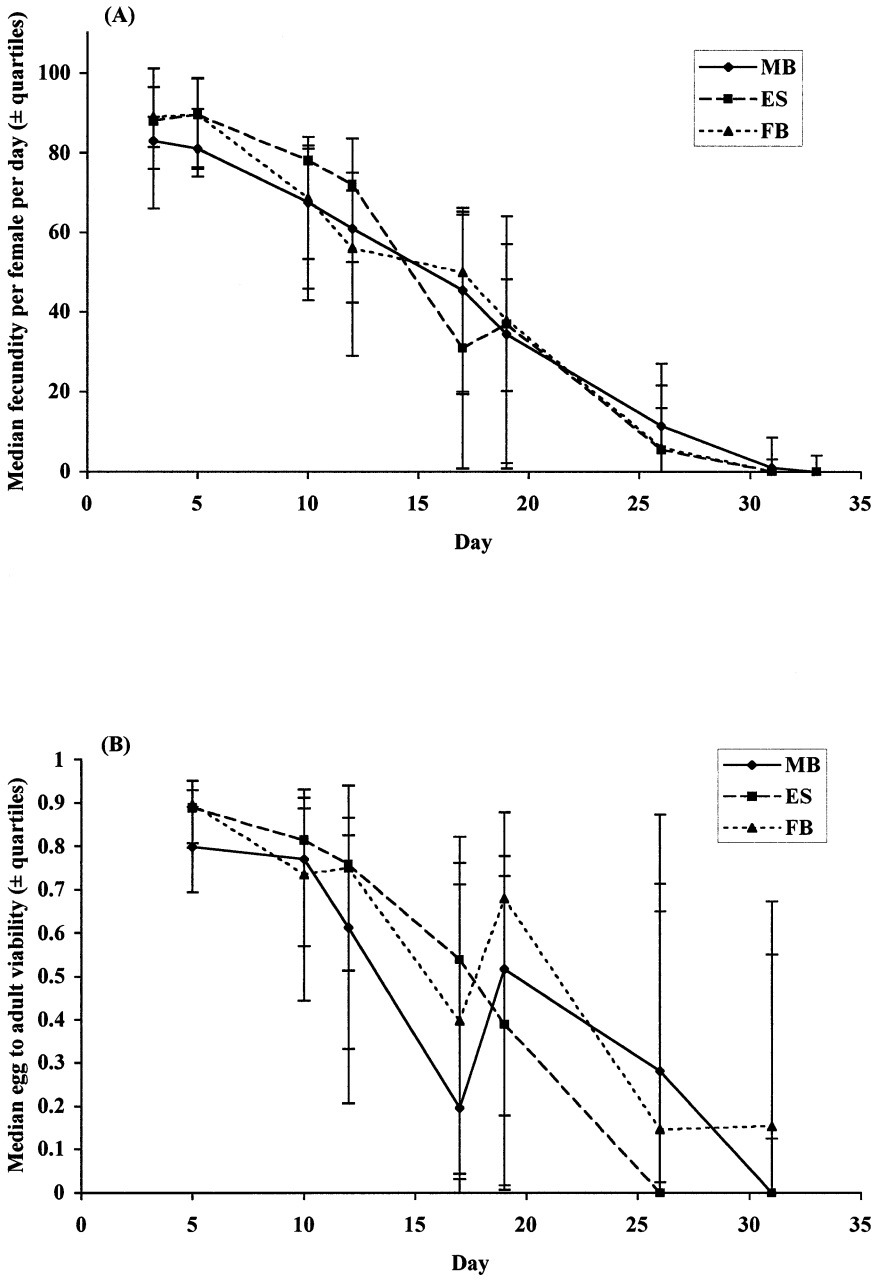


FIG. 3. (A) Median (± interquartile range) fecundity per female per day and (B) viability of eggs (number of progeny/number of eggs) of selection line females (replicates combined) continually housed with wild-type males.

selection treatments (Fig. 4, risk ratios, $\bar{X}$ and SE: MB = 1.09 ± 0.13, ES = 0.87 ± 0.13, FB = 1.11 ± 0.09; $F_{2,6}$ = 1.51, $P$ = 0.29). Mean survival values (days ± SE) were: MB1, 22.31 ± 0.59; MB2, 21.17 ± 0.56; MB3, 23.55 ± 0.59; ES1, 22.24 ± 0.58; ES2, 23.90 ± 0.69; ES3, 24.28 ± 0.62; FB1, 21.63 ± 0.58; FB2, 22.17 ± 0.56; FB3, 22.78 ± 0.61. There were significant differences between selection treatments in the total number of matings per female ($F_{2,6}$ = 12.12, $P$ = 0.008). Females housed with MB males mated significantly more frequently than females housed with ES males ($\bar{X}$ and SE: MB = 0.065 ± 0.007 matings per female, ES = 0.039 ± 0.003 matings per female; $P$ = 0.006). Females housed with MB males also mated more, but not significantly more, than females housed with FB males ($\bar{X}$ and SE: FB = 0.053 ± 0.008 matings per female; $P$ = 0.063). Females housed with FB males mated significantly more than females housed with ES males ($P$ = 0.039). However, these mating frequency differences did not result in significant differences in longevity. There were no significant differences between the selection treatments in the total number of courtship events received per female ($F_{2,6}$ = 2.36, $P$ = 0.18). There also were no significant differences in age-specific fecundity (Fig. 5A) between treatments on any day ($\chi^2_8 < 10.76$, $P > 0.21$, critical $P$ = 0.0083) or in egg-to-adult viability (Fig. 5B) between treatments on any day ($\chi^2_8 < 15.15$, $P > 0.056$, critical $P$ = 0.0083). The results show that, contrary to ex-

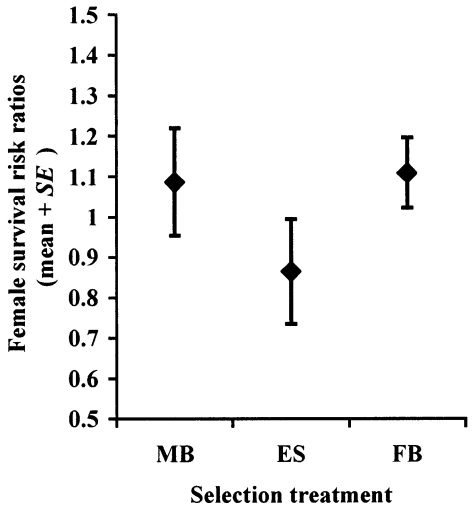


FIG. 4. Survival risk ratios (mean ± SE) of wild-type females continually housed with selection line males. Note that high risk ratio values indicate low survival.

pectation, there were no differences in the ability of selection males to harm wild-type females. This suggests that the evolution of female resistance was achieved by a response in females to differences in the number of matings received and not to differences between males in their ability to harm females.

### *Body Size, Accessory Gland, and Testis Size of Selected Flies*

There were no significant differences between the different selection treatments in any of the morphological characters measured. There were no significant differences between selection treatments in mean male (cube transformed, $F_{2,6} = 0.79$, $P = 0.50$) or female wing areas (Table 1, $F_{2,6} = 0.12$, $P = 0.89$). There were also no significant differences between selection treatments in mean male accessory gland area (Table 1, $F_{2,6} = 0.50$, $P = 0.63$) or mean testis area (Table 1, $F_{2,6} = 2.21$, $P = 0.19$). Nor were there any significant differences in allometry between selection treatments in mean accessory gland and wing area (log transformed, $F_{2,6} = 0.27$, $P = 0.77$) or mean testis and wing area ($F_{2,6} = 1.33$, $P = 0.33$).

## DISCUSSION

The experimental evolution imposed by the alterations in adult sex ratio manipulated sexual conflict as predicted. Females were courted and mated most frequently in MB populations, at intermediate frequency in ES populations, and least in FB populations. Thus, during selection, females from the MB lines should have experienced significantly greater male-induced harm than females from ES lines, with FB females experiencing the least harm. The most striking finding from this study was that, as predicted by sexual conflict theory, females evolved in response to differences in the risk of male-induced harm. Females from the MB lines showed significant and repeatable increased longevity in the presence of wild-type males compared to FB females, with females from the ES treatments showing intermediate longevity (Fig. 2). No such differences in survival were detected in the absence of males, consistent with the response in females being specific and directed towards altering resistance to male-induced harm. Thus, higher intensities of sexual conflict selected for more resistant female phenotypes and vice versa. These differences in female survival occurred in spite of, rather than being confounded by, inbreeding (which can result in decreased fitness (Sharp 1984; Miller et al. 1993; Garcia et al. 1994)) and can represent a problem if experimental and inbreeding predictions are the same (Holland and Rice 1999; Hosken et al. 2001). In our lines, the highest effective population size was in the ES, then FB, then MB lines (Wright 1938). In our tests, female survival was highest in MB, followed by ES, then FB females, a pattern opposed by inbreeding differences. The evolution of female resistance to male-induced harm in this study was not accompanied by detectable trade-offs in other fitness-related traits. Such costs of resistance are expected and it will be interesting to examine other components of fitness in these females.

Our results showed, contrary to our expectation, no evidence that males from the MB treatments had evolved to become more harmful to females. This suggests that females from these lines responded predominately to differences in mating frequency between the sex ratio treatments, and not to differences between the harmfulness of males. It will be interesting to confirm this by examining the responses of females from these lines to males with deficient ejaculate components (e.g., Chapman et al. 2003a; Liu and Kubli 2003).

Large *D. melanogaster* males are observed to be more harmful to females than are small males (Pitnick and García-González 2002). The lack of significant body size differences between selection treatment males in this study is therefore consistent with the idea that the evolution of female resistance was to differences in the number of matings received and not to differences between males in their ability to harm females. We also detected no significant differences in male testis or accessory gland size between treatments. This is perhaps unexpected, in light of the fact that the intensity of sperm competition differed between the sex-ratio treatments (being most intense in the MB lines, intermediate in ES lines, and least intense in FB lines). Male mating frequency also differed between sex ratio treatments (being highest in the FB lines, intermediate in ES lines, and lowest in MB lines). Therefore some or all of these traits would perhaps be expected to respond to these differences (see Hosken et al. 2001; Pitnick et al. 2001). For example, males from the FB lines would be expected to invest more in sperm production and males from the MB lines to invest more in Acp production and body size. However, it is difficult to make robust predictions of the direction of responses in these traits to differential sperm competition risk. Male strategies will vary depending on the exact level of competition, for example, it may pay a male to increase investment in traits that ensure sperm competitive success (e.g., large body size, large testis size, or large accessory gland size (Parker 1998)). However, this would only hold up to a certain level of competition, beyond which the risk of sperm usurpation would be so high that increased investment is selected against (Parker et al. 1996, 1997).

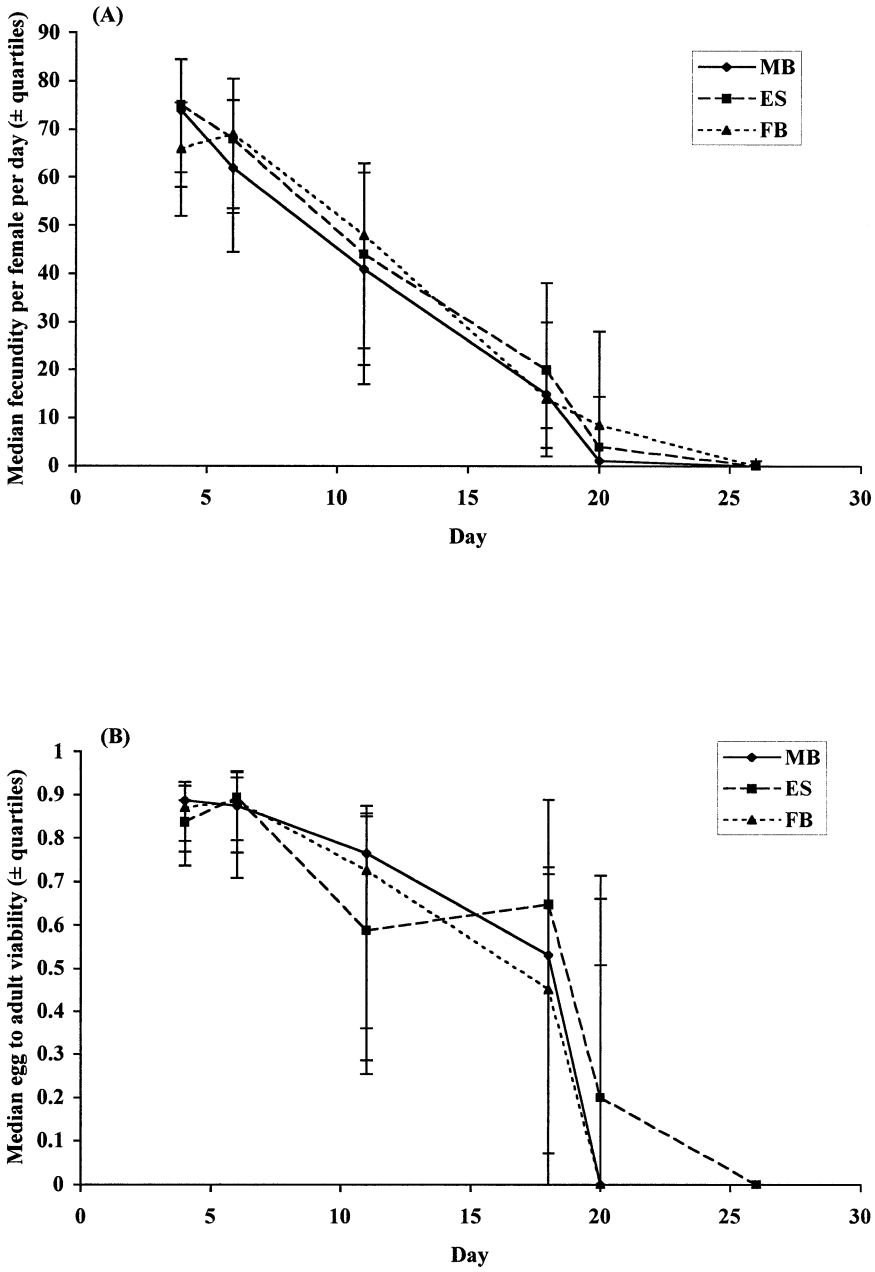


FIG. 5. (A) Median (± interquartile range) fecundity per female per day and (B) viability of eggs (number of progeny/number of eggs) of wild-type females (replicates combined) continually housed with selection males.

Under such potentially conflicting selection pressures, it is unclear whether the prediction is for larger or smaller body/accessory gland/testis size under the MB, ES, or FB regimes. Accurate predictions rely on identifying the exact selective pressures involved. For example, males from FB lines may exert similar amounts of energy in their frequent courtship bouts and matings as males from MB lines exert in male-male competition and their less frequent matings. Thus, in-

TABLE 1. Body (wing) sizes, accessory gland sizes, and testis sizes of the selected flies. Mean area ± SE.

| | Mean area ($mm^2$) ± SE | | | |
|---|---|---|---|---|
| Selection treatment | Female wing | Male wing | Accessory gland | Testis |
| MB | 1.334 ± 0.023 | 1.043 ± 0.016 | 0.231 ± 0.013 | 0.146 ± 0.005 |
| ES | 1.343 ± 0.011 | 1.063 ± 0.003 | 0.232 ± 0.002 | 0.165 ± 0.010 |
| FB | 1.330 ± 0.020 | 1.049 ± 0.014 | 0.242 ± 0.005 | 0.151 ± 0.004 |

vestment in competitive activity and body size could constrain the evolution of larger accessory glands. It would be interesting to probe for potential alternative strategies employed by males from the different selection lines (e.g., single mating productivity, induction of female nonreceptivity, ejaculate replenishment, and changes in sperm characteristics).

Lack of responses to selection could be explained if all flies were released from selection for harm and counterharm upon introduction to the novel selection line culturing regime. However, in the stock populations, under cage culture, selection on early fecundity is expected, if anything, to be even stronger than in the selection lines and adult life to be relatively short. This would not then relax selection in the manner proposed. In addition, this argument would not explain why females responded strongly to differences in mating rate, but in males a potential response in harming ability was masked by adaptation to the culturing conditions (which both sexes encountered). We therefore conclude that responses in males did not include differences in harming ability per se.

In summary, our results provide evidence that sexual conflict can be experimentally manipulated and that females can adapt to alterations in the intensity of sexual conflict by evolving increased or decreased resistance to male-induced harm. Our results also make further predictions. For example, ''susceptible'' females from the FB lines would be expected to alter their current reproductive output according to their residual reproductive value (Fisher 1930), and hence increase investment into current as opposed to future reproduction. In wild populations, one would also expect selection pressure in favor of the evolution of female resistance to male harm to track closely the operational sex ratio. Further work is now required to investigate this, to confirm the mechanism by which females evolve resistance to male-induced harm, to investigate the direction of responses relative to the base stock, and to elucidate whether males harm females to gain direct fitness benefits or whether females are harmed as a side-effect of male adaptations that confer increased reproductive success.

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