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Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load

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ABSTRACT **Although sexual selection can provide benefits to both sexes, it also can be costly because of expanded opportunities for intersexual conflict. We evaluated the role of sexual selection 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 promiscuous controls. Monogamous mating constrains the reproductive success of mates to be identical, thereby converting prior conflicts between mates into opportunities for mutualism. Random mate assignment removes the opportunity for females to choose beneficial qualities in their mate. The mating treatments were maintained for 47 generations, and evolution was allowed to proceed naturally within the parameters of the design. In the monogamous populations, males evolved to be less harmful to their mates, and females evolved to be less resistant to male-induced harm. The monogamous populations also evolved a greater net reproductive rate than their promiscuous controls. These results indicate a potentially widespread cost of sexual selection caused by conflicts inherent to promiscuity.**

A large body of theoretical and empirical evidence indicates that sexual selection can provide a variety of benefits to females (reviewed in refs. 1 and 2). Laboratory populations of *Drosophila melanogaster* have proven to be a valuable model system for measuring such benefits, e.g., improved survival (3–5). However, sexual selection is also demographically costly, reducing male viability through encumbering traits (6, 7). The costs of sexual selection that apply to females and those that arise from intersexual conflict have not been studied as thoroughly as the benefits. Theory and experiments indicate that some sexually selected traits increase male fitness at the expense of females (7–31). *D. melanogaster* has proven to be a valuable model here as well: (*i*) females experiencing experimentally reduced courtship (19) or mating rates (18) survive longer than controls but reproduce at the same rate; (*ii*) seminal-fluid components increase the competitive ability of accompanying sperm (32, 33) but also increase the mortality rate of inseminated females (27); and (*iii*) net male fitness increases at the expense of female survival when females are artificially prevented from coevolving with males (30).

Conflict between mates hinges on sexual infidelity. Under strict, life-long monogamy, any trait that lowers the reproductive success of one's mate lowers one's own reproductive success equally. Alternatively, whenever an individual has multiple mates, the lifetime reproductive success of that individual will differ from the success of its mates. Thus, promiscuity necessarily introduces the opportunity for sexual conflict through the evolution of novel traits that increase the reproductive success of members of one sex at a cost to members of the opposite sex.

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We replaced the naturally promiscuous mating system of *D. melanogaster* with enforced monogamy and random mate assignment in replicate populations. This treatment removes the opportunity for both intersexual and intrasexual selection. Therefore, we expected that natural selection would favor those individuals who are less harmful to their mate, indicating the variety and strength of conflicts that remained in the promiscuous control populations.

The treatment and control populations were given the opportunity to diverge for 32 generations before we began a series of assays. We measured the level of male-induced harm to females, as well as changes in specific male traits that were expected *a priori* to have contributed to a reduction of male-induced harm. If males evolve to become benign toward females, female defense against such harm becomes obsolete and will be selected against if it interferes with other components of female fitness. Therefore, we also assayed the level of female resistance to male-induced harm. Finally, we assessed the net reproductive rate of the populations under both mating environments. This measure evaluates the extent to which the removal of sexual selection has affected the ability of the populations to propagate themselves.

MATERIALS AND METHODS

Experimental Protocol. The ancestral population of *D. melanogaster* was established in 1988 from 400 mated females collected by L. Harshman (University of Nebraska, Lincoln, NE) in central California. Subsequently, this population has been maintained under the following conditions: effective population size of >5,000; 25°C; cornmeal/molasses/killed-yeast medium; 12-h light:12-h dark diurnal cycle; and 14-day generation cycle. Our experimental protocol maintains these conditions except as noted otherwise below.

To begin the experiment, 220 females and 220 males were sampled from the ancestral population. The sample was divided equally into replicates (A and B) and cultured. On the ninth day after egg laying, virgin progeny were collected from each replicate culture. Half of the offspring were assigned to a monogamy treatment (114 females, each housed individually with one randomly assigned male), and the other half were assigned to a control treatment (three males per female; otherwise identical to the monogamy treatment). The difference in sex ratio between treatments is a natural aspect of sexual selection in this species: "Mating takes place at the feeding site, where arriving females are greeted by the courtship of an average of five wild-type males" (34).

The assignment to the treatments described above was performed on day 1 of a 14-day propagation cycle. All flies were collected 2 h after eclosion by using CO_2 anesthesia for 3–4 min. Then the sexes were combined according to treatment type and housed for 5 days in "interaction vials" (100 mm × 13 mm) containing 3 ml of medium. The flies matured and mated in the interaction vials but no progeny were

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retained. On day 6, all flies were transferred without anesthesia to fresh "culture vials" (identical to interaction vials but seeded with live yeast), where eggs were laid overnight to propagate the next generation. On day 7, the adults from each of the four newly founded populations were discarded, and their progeny were allowed to develop until the end of the 14 day cycle. On day 1 of the following cycle, the day of maximum adult emergence, progeny eclosing within an 8-h period were collected and pooled without anesthesia from 100 productive culture vials per population; 2 h after collection, virgin males and females from each population were anesthetized with CO_2 and randomly parceled into individual interaction vials, as described above, to begin the next generation. In subsequent generations, the above protocol was reiterated. The number of productive culture vials used to propagate subsequent generations varied (n = 100–114), but in every generation an identical number was used from each population. No manipulation of family size or other aspect of artificial selection was used. Initially, the treatments showed no evidence of producing different offspring densities: monogamy, 19.4 ± 3.6; control, 17.2 ± 2.6; $P = 0.5$; Student's t test, $n = 4$, df = 2; densities were estimated in the third generation by the number of eggs per vial; 20 vials per population. Compared with the ancestral population, our protocol caused both treatments to have a lower density of adults and larvae.

Male Effect Assays. *Overview.* After 34 generations of enforced monogamy, two assays were performed to look for differences in the harm induced by males on their mates. One assay measured the net direct effect of experimental males on adult female fitness; the other focused specifically on the effect of seminal fluid on female survival. The assays used test females from outside the experimental populations to resolve the changes in experimental males independently from the changes in experimental females. Test females [C (1) DX *y f*; T (2.3) *rdgC st ri* p^p bw^D] carried a compound X chromosome and a multiply marked homozygous-viable translocation that had been repeatedly backcrossed through the ancestral population. The multiple genetic markers reduced the vigor of the test females, thereby increasing their sensitivity to detect differences in male-induced harm. To generate a sufficient number of experimental males for the assay, 60 parental females from the generation preceding the assay were cultured a second time in "assay vials" (95 mm × 27.5 mm with 10 ml of food and live yeast) to produce a replicate set of offspring. Egg density was adjusted within each vial by removing excess eggs to maintain uniformity with the egg density observed during normal propagation of the lines.

Net reproductive rate of adult test females. The net reproductive rate of adult test females was measured by an index incorporating both female survival and fecundity. Virgin test females were transferred without anesthesia into assay vials with experimental males. Each assay vial contained 20 2-day-old males and 12 5-day-old females (16 vials per population). The adults were transferred daily without anesthesia to fresh vials. Dead females were scored at each transfer. Average adult female fitness was measured as the cohorts' total egg production over the assay. In this assay of cohort productivity and in those described below, measurements were taken until test-female survival declined to 50% (29). To allow populations to be compared over identical time periods, measurements were truncated for all four populations at the point when the first population reached 50% mortality (3 days after initiating the assay). After each transfer of test females to fresh vials, the deposited eggs were transferred to 100-μm nylon filters by using a fine brush and tap water. To quantify the total egg mass, the collected eggs were boiled in deionized water for 20 s to dissolve particles of medium, rinsed in deionized water, and then dried overnight at 60°C before weighing.

Survival and fecundity of test females mated once. To assess divergence in the mortality effect of seminal-fluid components, virgin test females were allowed to mate once with experimental males. The males were then removed, and the survival and fecundity of the test females was measured. This assay was identical to the assay described in *Net reproductive rate of adult test females*, except that 100 min after being combined with the test females, the experimental males were removed by using CO_2 anesthesia for 45 s. The 50% mortality point was reached 22 days after mating. Copulation frequency was measured during the exposure period; 20 min after combining the sexes, most of the females were simultaneously *in copula*, and the frequencies between treatment were virtually identical: monogamy, 0.81 ± 0.025; control, 0.83 ± 0.042. After the initial pulse of mating, the copulation frequency decreased rapidly to 0 (by 70 min). No copulation occurred during the final 30 min. The above pattern indicates that after mating once, the females remained refractory for the duration of male exposure, consistent with previous studies (35) and our unpublished observations of the ancestral population. Per capita female fecundity was measured (as described in *Net reproductive rate of adult test females*) during the first 2 days after insemination. This assay encompasses the period when seminal fluid is known to influence this trait and when sperm stores remain unexhausted (36). Per capita fecundity was calculated as the total egg mass produced divided by the sum of the number of females surviving over day 1 and day 2 of the assay.

Courtship Rate. Adults used in this assay were sampled from the pool of virgin adults produced on day 1 in generation 45. On 12 occasions during the day of culture, each separated by at least 25 minutes, the presence of courtship was recorded for 42 pairs per population. Courtship was defined as male circling, wing vibration, licking, pursuit, or copulation attempt (37). Because male courtship behavior could potentially be affected by the presence of other males, as well as novel females, courtship rate was measured for individual males paired with single females from the same experimental population.

Female Resistance. *Survival when continuously exposed to males.* Adults used in this assay were sampled from the pool of virgin adults produced on day 1 in generation 32. Within each replicate, control or monogamy females were combined with control males in assay vials (20 males and 20 females per vial; 10 vials per population). The adults were transferred without anesthesia to fresh assay vials at least every second day. Dead adults were scored at each transfer. The assay duration was defined as described above in *Net reproductive rate of adult test females*.

Proximate fecundity rate after a single mating. To generate a sufficient number of experimental females for the assay, 60 parental females from the generation preceding the assay were cultured a second time (in assay vials) to produce a replicate set of offspring. Egg density was adjusted within each vial by removing excess eggs to maintain uniformity with the egg density observed during normal propagation of the lines. Virgin females from each replicate set of offspring were combined without anesthesia into assay vials with virgin ancestral males (i.e., males from the base population used to begin the experiment). Each assay vial contained 30 2-day-old males and 20 5-day-old females (9 vials per treatment). Virgin monogamy and control females were mated once during 100 min of exposure to males (as described above in *Survival and fecundity of test females mated once*). The males were removed by using CO_2 anesthesia for 45 s, and then the females were transferred to a fresh assay vial. The females were immediately permitted to lay eggs in the assay vials for 24 h. Per capita fecundity was measured (as described above in *Survival and fecundity of test females mated once*) over 24 h immediately after insemination, when any deleterious effects of seminal fluid on this trait should be most pronounced (36). In this assay, proximate fecundity replaced survival as an index of

female harm, because wild-type females mate many times before showing a mortality effect.

Net Reproductive Rate of Experimental Populations. These assays were performed with all populations housed under identical conditions (monogamy protocol in generation 45 and control protocol in generations 46 and 47). During generations 45 and 46, adults used in the assay were sampled from the pool of virgin adults produced during normal culture. In generation 47, assay adults were obtained from the progeny of the assay of generation 46. In each case, sample size was 114 vials per population per assay. The measure of net reproductive rate was the number of adult progeny produced per female. Only progeny that were available for collection during the normal culturing protocol were counted (i.e., those eclosing by day 1). This measure includes the fecundity of the females and the viability and development rate of their offspring. An additional two measures were made: development rate, the fraction of the total adult progeny that was available for collection during the normal culturing protocol (i.e., those eclosing by day 1), and total surviving adult progeny, the total number of adult progeny produced per female (i.e., including the slow-developing progeny that emerged after day 1).

Statistical Analysis. Student's *t* tests are used to evaluate all measures. A normal distribution of the data can be inferred, because each measure is an average (or a total) over a large number of contributing individuals. When the direction of a test could be prescribed *a priori*, a directed (38) as opposed to a one-sided test was employed. In all of our analyses, the statistically independent data are the population measures from each replicate treatment ($n = 2$ experimental + 2 control populations), rather than the individual flies or vials of flies that generate these population parameters. As a consequence, all measures of dispersion and tests of statistical significance are based on variation among replicated populations. Although this analysis has low statistical power, owing to the small sample size ($n = 4$), it avoids the problem of nonindependent data associated with pseudoreplication.

RESULTS

The net reproductive rate of adult test females was greater when they were housed continuously with monogamy males compared with control males (Fig. 1). Survival of test females was greater when mated once to monogamy males compared with control males, and this effect was not caused by differences in fecundity (Fig. 1). Monogamy males courted less frequently than control males when males were housed with the females with which they had evolved (Fig. 2).

Monogamy females die faster than control females when housed continuously with control males (Fig. 3). Male survival (not shown) did not differ between female treatments ($P = 0.9$, Student's *t* test, $n = 4$, df = 2; monogamy, 0.62 ± 0.068; control, 0.63 ± 0.013). Fecundity, over the first 2 days, after mating once to ancestral males, was reduced in monogamy females compared with control females (Fig. 4).

The net reproductive rate of the monogamy populations was greater than that of the controls (Fig. 5*a*). Development rate was significantly faster in the monogamy populations (Fig. 5*b*), whereas the total number of surviving progeny did not differ significantly (Fig. 5*c*).

DISCUSSION

Male-Induced Harm to Females. Previous studies have indicated that males can harm females through the toxicity of their seminal-fluid proteins (27) and through reduced female survival in response to persistent male courtship (19). The harm produced by seminal fluid is thought to be an incidental by-product of the beneficial aspects of seminal-fluid proteins: they mediate sperm competition (32, 33), and some of the proteins enter the female's circulatory system, where they influence her neuroendocrine system in ways that benefit the male (36). Recently, the harm of seminal fluid to females has come into question, however, because the toxicity of seminal-

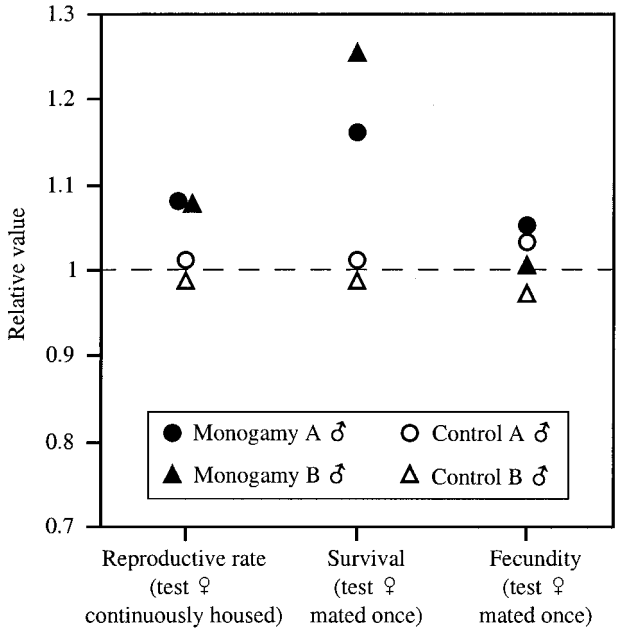

FIG. 1. The net reproductive rate of adult test females was greater when they were continuously housed with monogamy males compared with control males ($P = 0.01$); control mean = 30 mg eggs per cohort. Test-female survival was greater when mated once to monogamy males compared with control males ($P = 0.03$); control mean = 0.45 surviving females. Per capita fecundity of once-mated females was unaffected by the type of mate ($P = 0.3$); control mean = 67 μg of eggs per surviving female per day. All *P* values were calculated from directed Student's *t* tests (37); for each test, $n = 4$, and df = 2. To permit comparison across assays, the mean value of the control replicates was standardized to 1 in each case. Statistical analyses were conducted by using population values as data ($n = 2$ experimental + 2 controls), and dispersion within treatments is denoted by the spread between the two replicated populations (A and B) per treatment (see *Materials and Methods* for details).

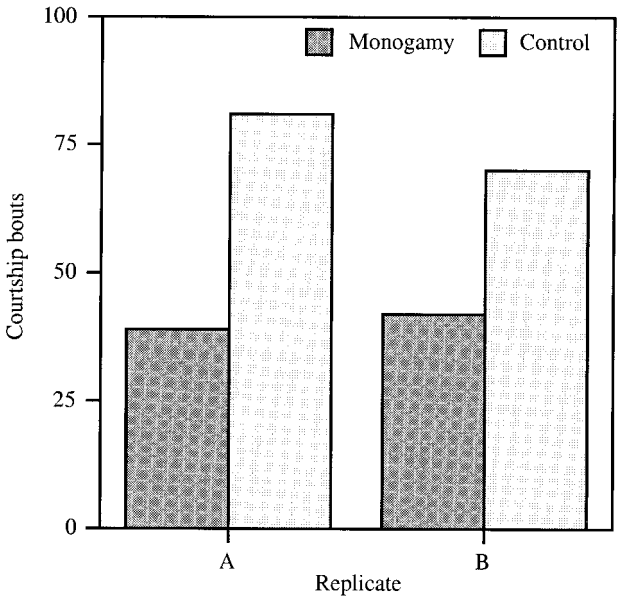

FIG. 2. Courtship rates of control treatments were greater than those of monogamy treatments ($P = 0.02$, directed Student's *t* test, $n = 4$, df = 2).

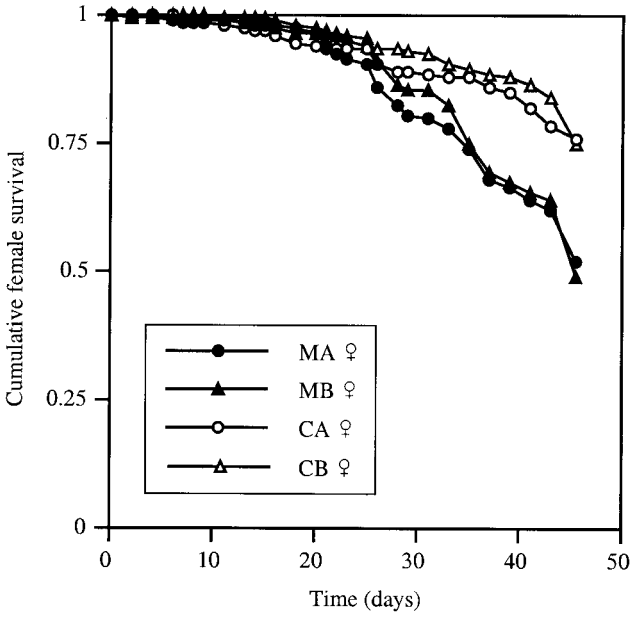

FIG. 3. The mortality of monogamy (M) females was greater than that of control (C) females when females were housed continuously with control males ($P = 0.003$, directed Student's t test, $n = 4$, df = 2). A and B refer to replicate populations.

fluid proteins is detectable only when females are reared under high nutrient conditions (39). Our evidence that the toxicity of seminal fluid is diminished when males evolve in the absence of sexual selection strongly supports the conclusion that the toxicity of seminal fluid is a sexually antagonistic trait (i.e., beneficial to males in the context of sexual selection but costly to females). Our additional finding that the elimination of sexual selection leads to the evolution of a substantially reduced rate of male courtship is also consistent with the hypothesis that mating displays can be sexually antagonistic.

Female Resistance. If male courtship and seminal fluid are costly to females and if this cost is a consequence of the operation of sexual selection, then (*i*) females should evolve

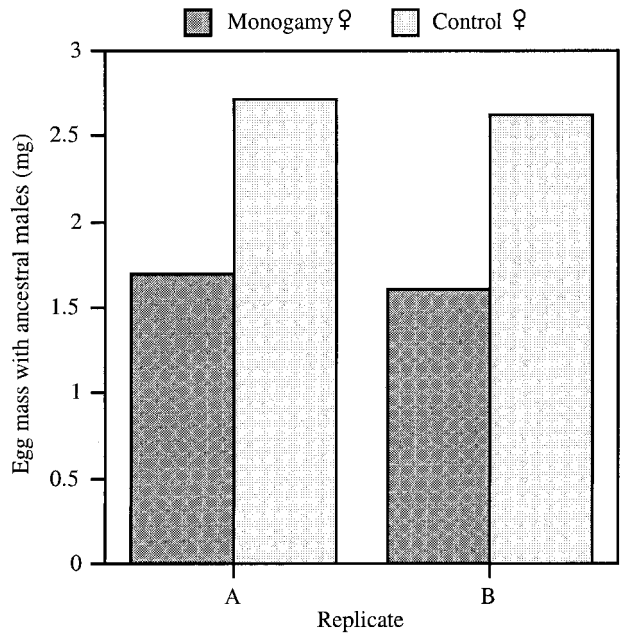

FIG. 4. Egg production by monogamy females was lower than that of control females when they were mated once to ancestral males ($P = 0.004$, directed Student's t test, $n = 4$, df = 2).

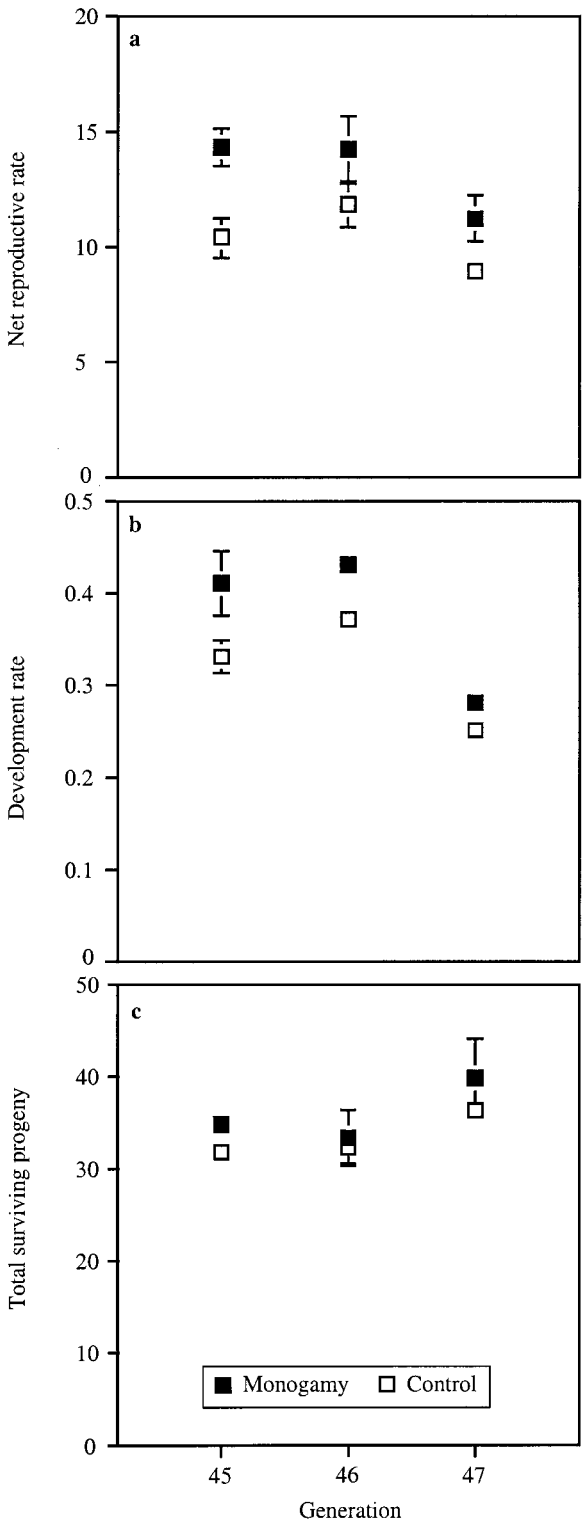

FIG. 5. Measures of population performance under standardized culturing protocol replicated across three generations. (*a*) The net reproductive rate is the number of mature (eclosed) progeny per female. This rate was counted at the end of each 14-day propagation cycle (monogamy > control; $P = 0.02$). (*b*) The development rate is the fraction of the total surviving progeny that had matured by the end of each 14-day propagation cycle (monogamy > control; $P = 0.006$). (*c*) Total surviving progeny included the slow-developing progeny that matured after the end of the 14-day cycle (monogamy = control; $P = 0.4$). Error bars are ±1 SEM (based on the variance between replicate populations; some error bars are not visible). For each graph, three 2-tailed Student's t tests ($n = 4$, df = 2) were pooled for each generation via a consensus combined-probability test (45) to obtain an overall P value.

counteradaptations (resistance) that reduce any harm inflicted by males, and (*ii*) the elimination of sexual selection should make such adaptations obsolete when males evolve to become more benign to their mates, as occurred in the monogamy treatment. If we assume that at least some of the genes that adapt females to male-induced harm also pleiotropically reduce female fitness in other ways, then female resistance should decline in the monogamy treatment. We found that females from the monogamy populations were harmed to a greater extent, by both ancestral males and control males, than females from control populations, providing strong evidence for the evolution of reduced female resistance.

The Load of Sexual Conflict. Collectively, the assays of male-induced harm and female sensitivity to male exposure are consistent with the hypothesis that sexual selection promotes antagonistic coevolution between genes influencing male mating success and female resistance. An alternative test of this hypothesis concerns the net performance of populations with and without sexual selection. To the extent that monogamy has removed a sexually antagonistic load from the populations, the populations' net reproductive rate should improve. Conversely, the monogamy populations should experience reduced fitness because of the loss of the beneficial effects of sexual selection (e.g., adaptive female choice). Also, in this specific experimental design, the monogamy populations may have experienced greater inbreeding because of fewer reproducing males. The greater net reproductive rate of the monogamy populations shows that the cost of sexual selection exceeded its potential benefits and thereby maintained a measurable load on the promiscuous populations. It is important to stress that our experimental results do not indicate that sexual selection necessarily harms a population more than it helps. Different environmental conditions may change the relative costs and benefits. However, it is clear that promiscuity introduces a conflict between the sexes, and as a consequence, sexual selection contains an intrinsic cost to females, maintained by antagonistic coevolution between the sexes.

Sexual Selection and Female Choice. The evidence presented here for the evolution of female resistance to sexually selected traits (e.g., seminal fluid or courtship) motivates a reevaluation of the factors that drive coevolution between male sexual traits and female choice. Previously, it has been proposed that seminal-fluid components may, like male courtship displays, be selected through female choice for "good genes" or material benefits (2). Heritable variation in a female's ability to affect the outcome of sperm competition has been shown (40, 41). In the most studied model, *D. melanogaster* (36), it now seems that some seminal-fluid components (e.g., proteins) inflict a direct cost to females through toxicity and, potentially, through the manipulative effects on the females' neuroendocrine systems (42–44). The evolution of female resistance to a seminal-fluid protein may interfere with its function, consistent with this study and previous work (30). These observations motivate an additional explanation for the inferred role of females in affecting sperm competition. Female choice of sperm may be a by-product of female defense against male-induced toxicity and neuroendocrine manipulation. A seminal-fluid protein, against which a female is less able to defend herself, may be favored selectively by virtue of being less encumbered by the female's counteradaptations.

Is the coevolutionary cycle between exaggerated male-display traits (e.g., male courtship dance and song in *Drosophila*) and female attraction to those traits driven primarily by female preference for honest indicators of male quality or by female resistance to the cost of sensory exploitation? Finding the answer to this question requires a more complete evaluation of the *direct* effects of male-display traits on female behavior and physiology, similar to the studies that have been completed with seminal fluid. It is well established that females are attracted to many male displays (1), but at any point in time, process, female choice, or female resistance can generate a positive correlation between the expression of a male trait and the propensity of a female to select that trait (14).

Conclusion. The work presented here isolates sexual selection to evaluate its role in antagonistic coevolution between the sexes and its effect on net reproductive rate. This study was motivated by the prediction that intersexual conflict should be an important general consequence of sexual selection (7–31). Our results support this prediction and, when combined with previous work (1, 2), indicate that sexual selection is a mosaic of processes both beneficial and costly to females. The rapidity and variety of the observed changes in this experiment suggest that intersexual conflict is strong, involves multiple loci, and may consequently perpetuate antagonistic coevolution between the sexes. This form of intraspecific antagonistic coevolution is one example of a more general process of interlocus contest evolution (ICE); this process should select for a variety of social traits ranging from gamete recognition proteins to male ornaments (13, 14).

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