

SEXUAL SELECTION AND IMMUNE FUNCTION IN *DROSOPHILA MELANOGASTER*

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The evolution of immune function depends not only on variation in genes contributing directly to the immune response, but also on genetic variation in other traits indirectly affecting immunocompetence. In particular, sexual selection is predicted to trade-off with immunocompetence because the extra investment of resources needed to increase sexual competitiveness reduces investment in immune function. Additional possible immunological consequences of intensifying sexual selection include an exaggeration of immunological sexual dimorphism, and the reduction of condition-dependent immunological costs due to selection of 'good genes' (the immunocompetence handicap hypothesis, ICHH). We tested for these evolutionary possibilities by increasing sexual selection in laboratory populations of *Drosophila melanogaster* for 58 generations by reestablishing a male-biased sex ratio at the start of each generation. Sexually selected flies were larger, took longer to develop, and the males were more sexually competitive than males from control (equal sex ratio) lines. We found support for the trade-off hypothesis: sexually selected males were found to have reduced immune function compared to control males. However, we found no evidence that sexual selection promoted immunological sexual dimorphism because females showed a similar reduction in immune function. We found no evidence of evolutionary changes in the condition-dependent expression of immunocompetence contrary to the expectations of the ICHH. Lastly, we compared males from the unselected base population that were either successful (IS) or unsuccessful (IU) in a competitive mating experiment. IS males showed reduced immune function relative to IU males, suggesting that patterns of phenotypic correlation largely mirror patterns of genetic correlation revealed by the selection experiment. Our results suggest increased disease susceptibility could be an important cost limiting increases in sexual competitiveness in populations experiencing intense sexual selection. Such costs may be particularly important given the high intersex correlation, because this represents an apparent genetic conflict, preventing males from reaching their sexually selected optimum.

KEY WORDS: *Drosophila melanogaster*, experimental evolution, immune function, life history, sexual selection.

Sexual selection is a powerful evolutionary process affecting genetic variation across a wide range of traits that influence, directly and indirectly, the ability of individuals to successfully compete for the fertilization of gametes (Andersson 1994). The strength of sexual selection is often greater than that of selection for greater viability (Hoekstra et al. 2001) and the energetically expensive evolutionary exaggeration of secondary sexual characteristics promotes trade-offs with other components of fitness (Höglund and

Sheldon 1998). In particular, sexual selection is predicted to be an important evolutionary force affecting variation in immune system function (Sheldon and Verhulst 1996; Zuk and Stoehr 2002; Schmid-Hempel 2003; Lawniczak et al. 2007). Resistance to disease is an important component of fitness, and the energetic demands of maintaining an immune system and of mounting an immune response may preclude the investment of resources in sexual displays and behaviors.

The role of sexual selection in shaping patterns of disease resistance and immune function has been of interest to evolutionary biologists at least since the pioneering work of Hamilton and Zuk (1982). Three major hypotheses have emerged, which emphasize the potential importance of sexual selection in affecting evolutionary changes in immune system function. First, because the evolutionary elaboration of secondary sexual characteristics requires increased investment of resources, this investment is predicted to promote a trade-off with immune function. As a result, the most successful genotype may not be the most resistant to disease, but the genotype making the best compromise between the competing demands of immunological defense and reproduction (Antonovics and Thrall 1994; Sheldon and Verhulst 1996; Van Baalen 1998; Boots and Haraguchi 1999; Jokela et al. 2000; Zuk and Stoehr 2002; Schmid-Hempel 2003). Second, if variation for this evolutionary trade-off involves genes with sex-specific effects, sexual selection is predicted to promote the evolution of sexual dimorphism in immune function (Zuk 1990; Zuk and McKean 1996; Rolff 2002; McKean and Nunney 2005). Lastly, the immunocompetence handicap hypothesis (ICHH) predicts that individual variation in success in mate competition reflects underlying genetic variation in condition specifically related to immune function and pathogen resistance (Hamilton and Zuk 1982; Folstad and Karter 1992; Wedekind and Folstad 1994).

We tested these three hypotheses by examining both phenotypic and genetic correlations between mating success and immune function in *Drosophila melanogaster*. Genetic correlations were tested by examining the immunological consequences of experimentally induced sexual selection. Such selection experiments are a powerful approach for investigating trade-offs and other hypotheses concerning the direct and correlated response to selection (Rose 1991; Reznick 1992; Roff 2002). Sexual selection was induced in three replicate populations of *D. melanogaster* by creating a male-biased adult sex ratio at the start of each generation. After 58 generations of selection, we compared the sexual competitiveness and immune function of control (C) and sexually selected (SS) males and females. We established that males from sexually selected lines were more sexually competitive than control males. We then evaluated three predictions: first, the trade-off hypothesis prediction that this increased competitiveness would result in a reduced immune function; second, the sexual-dimorphism hypothesis prediction that sex differences would be exaggerated in sexually selected lines; and third, the ICHH prediction that sexual selection would cause a change in the condition-dependent expression of immunity by lessening the marginal cost of increased mating effort. Our results supported the trade-off hypothesis, because they clearly indicated a genetically based immunological cost of sexual selection, a cost reflected in the phenotypic correlation between mating success and immune function. However, we found no evidence that sexual selection promoted an exagger-

ation of sexual dimorphism or that sexual selection affected the condition-dependent expression of immunocompetence.

Materials and Methods

THE BASE POPULATION

The base population of *D. melanogaster* used in all experiments was established from about a 100 mated females caught in the University of California Riverside orange groves in 1995. This base population was maintained as a large (>1000 adults) outbred population in bottles for two years before being used in the experiments. It is necessary to use such a laboratory-adapted population to ensure that linkage disequilibrium generated during adaptation does not confound the selection procedure (Rose et al. 1996). This is particularly important in the present context because (1) the linkage disequilibrium could affect the magnitude and direction of the phenotypic correlation between immune function and sexual competitiveness in the unselected base population, and (2) it is possible that sexual selection could increase the rate of adaptation to the new environment (Lorch et al. 2003) possibly creating spurious genetic correlations among fitness traits.

PHENOTYPIC CORRELATES OF MALE MATING SUCCESS IN THE BASE POPULATION

In this experiment we investigated the phenotypic correlates of male mating success in our unselected base population. In particular we were interested in the relationship between sexual competitiveness and immune function and whether this relationship was affected by experimental manipulation of male condition. We define condition as the pool of resources that can be allocated among these fitness traits (Rowe and Houle 1996).

At the start of the experiment, males were classified as initially successful (IS) or unsuccessful (IU) based on their mating success in a mate choice experiment. Subsequently, the condition of these males was manipulated by placing them in vials either alone or with five females. This manipulation of the sexual environment affects male condition via a phenotypic trade-off between male sexual activity and both immune function and male weight (McKean and Nunney 2001, 2005).

Experimental flies were reared in low-density bottles established by placing 15 male and 15 female parents in dietary yeast-supplemented bottles for 24 h of egg laying. To initiate the experiment, adult flies were collected as virgins from a rearing bottle (day 0) and allowed to mature in groups of 25 flies per same-sex vial. On day 3, 50 males (from a single rearing bottle) and 50 females (from a different rearing bottle) were transferred without anesthesia into a 2-L flask that served as a mating chamber. The first 20 mated pairs (designated as initially successful or IS males) were gently aspirated from the mating chamber. Of the remaining males, 20 were randomly chosen for further tests (designated as initially unsuccessful or IU males).

Immediately following the removal of the last male from the mating chamber, IS and IU males were evenly split and placed individually in a vial either alone or with five previously mated females. The resulting experimental design is a complete factorial with 10 replicate vials (per block) for each combination of initial success and subsequent sexual environment. These females were four to five days old when the males were introduced. Vials were then randomly assigned a position in a tray and placed in an incubator at 25°C.

On days 5 and 7, the sexual behavior of the IS and IU males held with females was observed. Vials were placed on the bench top and 15 observations were made during a 10-h period with each observation separated by at least 30 min. Whether a male was observed courting or mating was recorded for each vial. Males housed alone in vials were handled similarly. Courtship behavior was never observed for males housed alone. At the end of the day, the flies were transferred to a new vial without anesthesia.

On day 9, the ability of males to clear experimental injections of *E. coli* D21 bacteria was assayed as described previously (McKean and Nunney 2001, 2005). Briefly, flies were injected with *E. coli* D21 suspended in a *Drosophila* Ringer's solution using a nanoliter injector fitted with pulled glass capillary needles. *Escherichia coli* D21 carries resistance genes to both ampicillin and streptomycin. After three days, each male was homogenized in a 1.5-mL centrifuge tube in 40 μ l of Ringer's solution. The homogenate was then diluted to 1 mL, and 100 μ l of the solution plated on LB agar plates containing 100 μ g/mL streptomycin. The plates were then incubated and scored for the number of *E. coli* D21 colonies using a colony counter. The clearance of *E. coli* D21 is due to induction of the humoral immune response and not simply due to flies being an unsuitable host for the bacteria (McKean and Nunney 2001).

Prior to homogenization, the right wing of each male was clipped, placed on transparent tape and adhered to a note card. Wing length was measured as the distance between the anterior crossvein to the distal end of the third vein. Measurements were performed on digital images using NIH Image version 1.62.

For each run of the experiment, all males were from the same rearing bottle, thus limiting nonexperimental sources of environmental variation in condition by eliminating among-bottle variation. The experiment was a complete factorial design with two levels: initial mating success (successful or unsuccessful) and sexual stress (males alone or males with five females). It was repeated four times, with the random effect of REPLICATE entered in statistical models. Each REPLICATE represented an independent sampling of the base population.

To examine the relationship between the effects of the initial mating success of males and of their subsequent sexual environment on immune function we used a mixed model analysis of covariance (ANCOVA) with the natural log of COLONY

COUNT as the dependent variable measuring immune function (higher COLONY COUNT corresponding to less effective immune function). The statistical model included the fixed effects of initial success (SUCCESS) and sexual environment (SEXENV) and their interaction along with the random effect of REPLICATE and wing length as a covariate. Interactions between wing length and the fixed effects were not significant ($P > 0.5$). Residuals from this model were examined for goodness of fit to a normal distribution to confirm that the assumptions of the tests were fulfilled.

Behavioral data on males housed with females were analyzed using the angular transformation (Sokal and Rohlf 1995) of the proportion of times a male was observed courting to meet the assumptions of analysis of variance (ANOVA)/ANCOVA. Repeated measures ANOVA revealed that although there was a significant overall decline in the amount of courtship from day 5 (males courting in 32% of observations) to day 7 (males courting in 21% of observations; $P = 0.0004$), this decline in courtship was similar for both IS and IU males (Initial Success \times Time, $P > 0.25$) so final analyses were done with ANOVA using data averaged across the two time periods.

LABORATORY-INDUCED SEXUAL SELECTION

Theoretical background

We induced sexual selection in three replicate lines of flies by increasing the sex ratio to just under three males to one female. Each selected line was paired with a control line representing three independent samples of the base population. Control lines were kept at a 1:1 sex ratio. Given this shift in sex ratio, sexual selection should act more strongly on males (Emlen and Oring 1977). More precisely, it is expected to increase the intensity of sexual selection (I , the standardized variance in the number of matings per male) (Wade 1979; Wade and Arnold 1980). Lottery polygyny provides a good approximation to the mating system of *D. melanogaster* so that $I = m/(nf)$, where m and f are the number of breeding males and females and n is the number of matings per female (Nunney 1993). Thus the experimental shift in sex ratio was expected to increase I about threefold (given $n = 1$, see below).

Selection procedure

The three replicates (I, II, and III) of paired sexually selected (SS) and control (C) lines were maintained for 58 generations prior to testing for a response to selection. Each replicate was established by randomly sampling 200 females from the base population and then randomly assigning the offspring from these females to the control line and the sexual selection line. Sexual selection lines were maintained with 50 females and 120 males each in three bottles ($N = 510$; $N_e \cong 256$) whereas control lines were maintained with 50 males and 50 females each in four bottles ($N = 400$;

$N_e \cong 267$). Estimates of the effective population size assume lottery polygyny with females singly mated ($n = 1$) during the 12-h oviposition. A greater degree of female remating ($n \rightarrow \infty$) increases the effective population sizes to 443 and 400, respectively (Nunney 1993). The paired control and sexually selected lines for each replicate were always handled in a similar manner and all lines were maintained at 25°C.

To start each generation of selection, virgin flies were collected from all bottles within each replicate and housed in same-sex vials until enough adults had been collected. An equal number of flies were collected from each bottle of a line, mixed and then the females and males (50 and 120 per selection bottle; 50 and 50 per control bottle) were transferred into bottles (three selection, four control) without anesthesia and returned to the incubator. After three days the flies were transferred into a set of egg-laying bottles supplemented with dietary yeast. After 16 h the flies were transferred into a second set of egg-laying bottles (supplemented with yeast) and then 12 h later the adult flies were discarded. Flies for the next generation were sampled from the second set of bottles, the first set acting as a back up. The total generation time was 14 days.

ANALYSIS OF THE RESPONSE TO SELECTION

Competitive male mating success

To test whether sexual selection had caused changes in sexual competitiveness, we allowed males from the paired control (C) and sexually selected (SS) lines to compete directly for matings with either C or SS females (from the same replicate population) in a mating chamber. Flies were collected from low-density bottles established by placing 15 males and 15 females in bottles provided with excess yeast and transferred every 24 h. In each run of the experiment, 20 three-day-old virgin males from each line (C and SS) were combined in a 2-L flask with 40 virgin C or SS females. Males and females of the same type were always collected from different bottles. SS and C males had been marked with different fluorescent dusts on the day prior to the mating chamber tests. The micronized fluorescent dusts were those previously tested by Crumacker (1974). The dust applied was rotated between trials. As mating began, mated pairs were gently aspirated into a vial and the time of mating was recorded. The first 20 mated pairs were collected and then the type of male determined by examination under a dissecting scope using a UV lamp. Twenty-six runs for each paired replicate population, 13 runs per female type, were performed.

For each run of the experiment, a chi-square value can be calculated from the 2×2 contingency table of male selection history (C or SS) and mating status (mated or unmated). The square root of this value, Chi, was given the sign of the difference in the cross products (positive if SS males out-competed C males) and this statistic was used in analyses. We used ANOVA with the chi score as the dependent variable to test for differences between

female type (C or SS), replicate population (I, II, or III), fluorescent dust, and all two-way and the three-way interaction.

A test of the null hypothesis of no difference in the competitive ability of C and SS males is accomplished by calculating the z-statistic of the chi-values as $z = \sum_{i=1}^g \chi_i / \sqrt{g}$ (where $g = 26$, the number of replicates per line) and comparing this value to the standard normal distribution (Everitt 1992).

Dry weight at emergence

To determine if the selection regime had caused changes in the body size of flies we determined the dry weight of males and females from the control and selection lines. Newly hatched larvae from each line and selection treatment were collected and placed 20 larvae per yeast-supplemented vial. Two vials for each line and selection regime were set up on each of three days for a total of six vials per line. Five flies of each sex were sampled within 24 h of emergence, immediately frozen, and then placed in a drying oven for 24 h prior to weighing. Analysis using ANOVA is based on the natural-log transformed vial means for each sex.

Development time

Because we found that sexually selected flies were larger than controls, we investigated whether this increased size came at a cost of also increasing development time. Newly hatched larvae were placed 20 larvae per vial in vials supplemented with excess dietary yeast. Three vials for each line and selection regime were set up on each of two days, representing a temporal block, for a total of six vials per line. After pupation vials were checked until the first adults began to emerge. Thereafter, vials were checked every 6 h and the number and sex of emerging flies was recorded. Data were analyzed using ANOVA.

Immune function and male condition

The immune function of virgin and sexually active C and SS males was assayed. As previously mentioned, this environmental manipulation directly affects male condition (McKean and Nunney 2001, 2005). In this experiment, 10 males were collected as virgins on day 0 from each of eight separate rearing vials established by placing 40 newly hatched larvae in vials supplemented with excess dietary yeast. Each group of males was placed in a fresh vial. An additional four to five males per rearing vial were immediately frozen, and then dried for later weighing for use as a covariate in an analysis of covariance based on vial means for weight and immune function. On day 2, the males were split into two treatment groups and placed singly in vials either alone or with five previously mated females from the laboratory base population. Sexual behavior was observed on days 3 and 4 (observation vial 1) and then flies were transferred into a new vial on day 5. Behavioral observations repeated on days 6 and 7 (observation vial 2). Behavioral observations consisted of 15 observations per day, each

observation separated by at least 30 min. Whether a male was observed courting or mating was recorded. Males alone in vials were handled similarly, but sexual behavior was never observed in these males.

All males were injected with *E. coli* D21 on day 8 and returned to a new vial, remaining either alone or with five females. On day 11, the males were homogenized and their bacterial contents were estimated.

The three replicate populations were tested on different days (designated as REPPOPDAY in the model). Therefore, any significant REPPOPDAY effects confound population effects with the inevitable between-day variation in the dose of bacteria injected and other sources of environmental variance. However, this design maximizes the ability to test for differences between C and SS flies within the paired replicate populations by eliminating variance introduced by testing flies across different days.

Data on the number of *E. coli* remaining per fly and behavior were analyzed using ANOVA. *Escherichia coli* counts were natural-log transformed prior to analysis to ensure that the assumptions of ANOVA were satisfied. Independent variables were the fixed effects of SELECTION (control or sexually selected) and sexual environment (alone or with five females, SEXENV in the model) along with the random effects of REPPOPDAY (I, II, and III), and rearing VIAL (nested within REPPOPDAY and SELECTION). The analysis with weight as a covariate was done using ANCOVA on data consisting of the vial means for weight and the number of *E. coli* remaining per fly. None of the interactions between WEIGHT and the fixed effects were significant ($P > 0.5$).

Data on sexual activity (the daily proportion of observation periods with courtship or mating) of each male housed with five females were first analyzed using ANOVA on the angular-transformed daily courtship of males averaged across the four days of behavioral observations. The angular transformation (arcsine square root) helps stabilize the variance of proportional data. Second, we used a repeated-measures ANOVA, creating a set of three orthogonal contrasts among the four days of repeated measures to test the separate effects of fly age and time spent in a new vial on male behavior. The first contrast (FLY AGE), tests the hypothesis that male behavior is changing with age (Contrast 1: Day 3 and Day 4 vs. Day 6 and Day 7). The second contrast (TIME IN VIAL), tests the hypothesis that sexual activity changes not with age, but with the time spent in a new vial (Contrast 2: Day 3 and Day 6 observations vs. Day 4 and Day 7 observations). The third model tests the FLY AGE \times TIME IN VIAL interaction (Contrast 3: Day 3 and Day 7 vs. Day 4 and Day 6). The independent variables SELECTION, REPPOPDAY (and their interaction) and rearing VIAL (nested within REPPOPDAY and SELECTION) were included in both models.

We also examined the relationship between observed courtship levels and immune function using ANCOVA on the sub-

set of the data of flies housed with five females. The transformed average daily proportion of times a male was observed courting (AVECOURT) was entered into the model along with the fixed factors of REPPOPDAY and SELECTION and the random factor of rearing VIAL. All interactions with the fixed effects and AVECOURT were nonsignificant ($P > 0.5$).

Sex differences in immune function

In this experiment we tested the immune function of virgin males and females from C and SS lines. Flies were collected as virgins (Day 0) from six separate rearing vials established in the same way as described in the previous experiment. Again, an additional five flies of each sex were immediately frozen and then later dried and weighed for use as a covariate in the analysis. Virgin flies were placed singly in vials provided with excess dietary yeast. On day 4, flies were injected with *E. coli* D21 and on Day 7 homogenized and the remaining number of bacteria was estimated. The experiment was carried out over three days, the effect of DAY representing a temporal block. All replicate populations were tested on each day, but again, to maximize our ability to observe between selection regime differences, the statistical effect of replicate population is confounded by the use of different needles, slightly different times of injection, and homogenization. Data on the natural log-transformed *E. coli* counts were analyzed using ANOVA. The model included the fixed factors of sexual selection history (SELECTION) and SEX, along with the random factors of replicate population (REPPOP) and DAY. ANCOVA on the vial means of the *E. coli* counts of each sex was used to determine effects independent of size variation. The model was same as above, but included the vial mean of the natural log of fly dry weight of each sex (WEIGHT) as a covariate (all interactions with the WEIGHT were nonsignificant).

Results

PHENOTYPIC CORRELATES OF MALE MATING SUCCESS IN THE BASE POPULATION

We examined three main questions in comparing males that were initially successful (IS) to those that were unsuccessful (IU) in the competitive mating test. Did successful males show superior immune function, indicating overall superior condition, or did they show inferior immune function, indicating extra mating effort that traded off with immune function? Was the immune function of IS and IU males differentially affected by the phenotypic manipulation of male condition? Were there differences in the sexual behavior of IS and IU males when subsequently housed with five females?

Manipulation of male condition by the subsequent placement of IS and IU males in vials either alone or with five females had the predicted effect of reducing male immune function ($P < 0.0001$).

Table 1. Antimicrobial immune function and sexual behavior of males from the unselected base population. Males were classified as “initially successful” (IS) or “initially unsuccessful” (IU) based on their success in a competitive mating trial. After the trial, male condition was manipulated by placing each male in a vial either alone or with five previously mated females. (A) Immune function. ANCOVA (with wing length as a covariate) of the *E. coli* counts (which were natural-log transformed for the analysis). Unlabelled numbers are *F*-values ($df=1, 136$). Similar results were obtained from ANOVA without the wing length covariate in the model (not shown) (B) Courtship. Results from Wilcoxon rank-sum test of the proportion of times a male was observed courting in vials with five females (Chi-square with $df=1$). (C) Mating. Differences between IS and IU males in their subsequent mating success in vials with five females. The *P*-value is from a two-tailed Fisher’s exact test of the 2×2 table of IS versus IU and the number of flies seen mating versus those never seen mating. (* $P < 0.05$, *** $P < 0.001$ for all tests). (D) ANOVA of wing length. Shown is the *F*-value ($df=1, 140$).

Dependent variable	Initial mating success	Sexual environment	Interaction	Wing length covariate
(A) Immune Function (with size covariate)	IS < IU 4.83*	Alone > 5 females 15.73***	1.61 (ns)	$\beta=0.012$ 0.60 (ns)
(B) Courtship Level	IS = IU 3.29 (ns)	–	–	–
(C) Proportion of Males ever observed mating	IS = IU $P=0.465$	–	–	–
(D) Wing length	IS = IU 1.82 (ns)	–	–	–

This manipulation had similar effects on both IS and IU males, as revealed by the nonsignificant interaction term ($F_{1,136} = 1.610$, $P = 0.207$). Overall it appeared that IS males cleared significantly fewer bacteria than initially unsuccessful males (IU) ($P < 0.05$, Table 1; Fig. 1) indicating poorer immune function. However, contrasts of the difference between IS and IU males within each sexual environment suggested that the significant main effect of initial mating success was driven primarily by differences apparent only when males were housed with five females (IS vs. IU, housed alone: $F_{1,136} = 0.431$, $P = 0.513$; housed with five

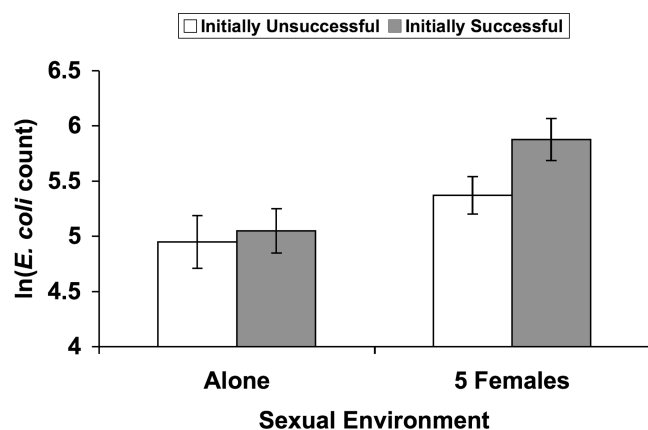


Figure 1. Male mating success and antimicrobial immune function. Males from the base population that were “initially successful” or “initially unsuccessful” in competition for mating were subsequently housed in vials either alone or with five females, representing a manipulation of male condition. High scores indicate poor microbial clearance, and are the least square means of $\ln(E. coli)$ remaining per fly \pm SE from a model including wing size as a covariate (see Table 1).

females: $F_{1,136} = 6.053$, $P = 0.015$). The size of the flies, as measured by wing length, did not differ between the IS and IU males, and was also not a significant predictor of variation in immune function (see Table 1).

In contrast to the lower immune function of IS males (vs. IU males) when housed with females, there was no significant difference in the level of courtship of IS and IU males when housed with five females; in fact, IS males were observed courting 23.5% of the time, which was less than the 29.8% of observations in which IU males were observed courting (a nonsignificant difference, $P = 0.063$, Table 1). This observation is contrary to the possibility that higher courtship in IS males was the cause of their lowered immune function. Despite having lower courtship activity, the proportion of IS males observed mating at least once was slightly greater than IU males (71% of IS males vs. 59% of IU males) but again the difference was not significant (Table 1).

All IS males were guaranteed to have mated at least once during the initial mating trial. We tested whether the difference between IS and IU males could be due to enhanced immune function in IU males that had never mated. The number of bacteria recovered from IU males that were never observed mating ($\bar{X} = 5.28 \pm 0.48$) was not significantly different from IU males that had been observed mating at least once ($\bar{X} = 5.43 \pm 0.47$; $F_{1,33} = 0.402$, $P = 0.53$).

LABORATORY-INDUCED SEXUAL SELECTION AND IMMUNE FUNCTION

Selection response: male sexual competitiveness

Our initial analysis compared the sexual success of the sexually selected (SS) and control (C) lines, when they competed for matings

with the test females, with each replicate outcome expressed as a single Chi score that summarized their relative success. Test females were either from the selected or control line (FEMTYPE: C or SS), and the color of the marker dust (DUST) was alternated between SS and C males. There was no significant effect of FEMTYPE or its interactions ($P > 0.25$), thus male mating differences were independent of the type of female. In addition, DUST had no influence of male performance ($P > 0.25$). However, the effect of replicate population (REPPOP: I, II, or III) was close to significance ($P = 0.061$), indicating that the populations differed in the relative performance of their SS and C line. To ensure that this population effect did not confound the differences between SS and C lines, all subsequent tests were split between the three independent populations, with scores combined across female selection history and fluorescent marker.

The analysis of the three populations showed that SS males were significantly more successful than C males in obtaining matings in the mating chamber (all $P < 0.01$, Table 2; Fig. 2). The effect of selection ranged from a 10% increase in mating success in replicate population I, to around a 20% increase in mating success in populations II and III.

Selection response: size and development time

The dry weight of SS flies of both sexes was greater than C flies ($P < 0.001$, Table 3A; Fig. 3). This difference in fly size also resulted in increased development time, with SS flies taking 3% longer to develop than C flies ($P < 0.001$, Table 3B).

In the analysis of development time, the replicate population \times selection interaction was significant, so the significance of the difference between control and sexually selected flies was examined within each replicate population. Within each replicate population control flies developed significantly faster than sexually selected flies, with the source of the interaction being the much smaller difference in REPPOP I. This population also had the smallest difference in sexual success of SS vs. C males (see above).

Table 2. Chi-square analysis of sexual competitiveness of control (C) and sexually selected (SS) males in a mating chamber. Males competed for matings, using either all C or all SS females. Positive values of chi-square test and z (the unit normal variate) indicated that SS males outcompeted C males, as predicted. The total number of trials within each replicate population was 26. All results remain significant after using a Bonferroni correction for multiple tests ($P < 0.01$).

Replicate population	$\sum \chi$	z	$P(z)$
I	15.68	3.07	0.0011
II	29.83	5.85	<0.0001
III	35.58	6.98	<0.0001

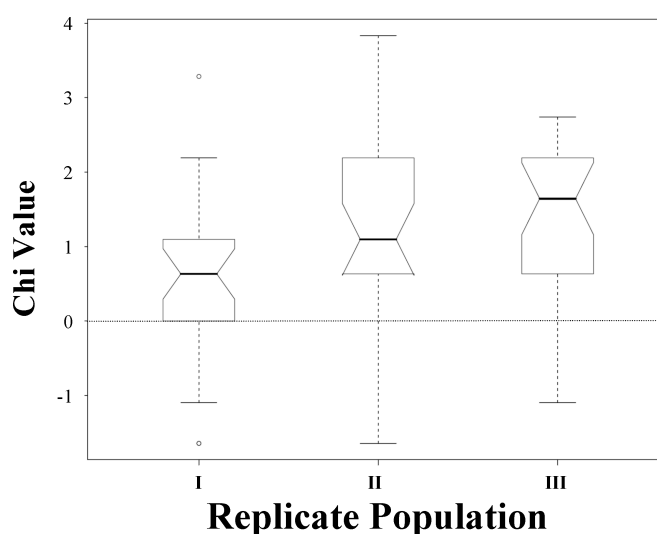


Figure 2. Box-and-whisker plot of the median signed Chi-values for the competitive mating success of sexually selected (SS) versus control (C) males. Values greater than zero indicate that the SS males were more successful than C males. Ends of the boxes represent the 25th and 75th quantiles, and the whiskers represent the range of the data. Points outside of the whiskers are possible outliers. The notches in the boxes are roughly equivalent to 95% confidence intervals for the median.

The highly significant effect of SEX in both size and development time analyses reflects the larger size and shorter pupal period of female *D. melanogaster* (Nunney 2007).

Selection response: immune function and courtship of males

Sexually selected (SS) males showed a reduced immunocompetence because they cleared significantly fewer bacteria than C males ($P < 0.001$, Table 4A; Fig. 4). In agreement with previous results, males with five females cleared significantly fewer bacteria than virgin males ($P < 0.001$), but there was no indication of any interaction between male selection history and sexual environment ($P > 0.25$).

The possibility that the differences in size between SS and C males was responsible for this difference in immune system function was tested by adding weight (using the vial means) as a covariate in the analysis. The effect of weight was not significant and did not affect the significance of the main effect of SELECTION (Table 4B).

The analysis of the behavior of C and SS males when they are housed with five females revealed a significant negative correlation between courtship and immune function, that is a positive regression between courtship levels and the number of *E. coli* D21 bacteria remaining per fly (slope $\beta = 0.642$, $P < 0.05$, Table 4C). However, this did not affect the significance of the main effect of SELECTION in reducing immune function ($P < 0.001$).

Table 3. Analysis of (A) adult dry weight and (B) egg-to-adult development time. The RP×SEL interaction was significant for the analysis of development time, therefore, the pairwise contrasts of control and sexually selected flies for each replicate population are shown. Estimates of sums of squares of random effects using the REML method of JMP are based on shrunken predictors, meaning that traditional tests of significance using *F*-tests are not appropriate. In this and subsequent tables, tests of random effects reflect whether the 95% confidence interval of the maximum-likelihood estimate of the effect is greater than zero (**) or includes zero (n.s.). Significance levels of $P < 0.05$ are bolded.

Source	df	SS	<i>F</i>	<i>P</i>
(A) Dependent variable: ln(Dry weight)				
SELECTION (SEL)	1	0.0411	12.73	0.0008
REPPOP (RP), Random	2	0.0242	–	**
RP×SEL	2	0.0021	0.32	0.7252
SEX	1	3.1333	971.08	<.0001
RP×SEX	2	0.0115	1.79	0.1769
SEL×SEX	1	0.0008	0.25	0.6205
RP×SEL×SEX	2	0.0016	0.24	0.7843
BLOCK, Random	5	0.1255	–	**
ERROR	55	0.1775		
B) Dependent variable: Development Time				
SEL	1	3261.26	153.13	<.0001
RP	2	448.82	–	**
RP×SEL	2	643.49	15.07	0.0005
REPPOP I (C vs. SS)	1	161.94	7.58	0.0175
REPPOP II (C vs. SS)	1	2169.32	101.59	<.0001
REPPOP III (C vs. SS)	1	1567.26	73.39	<.0001
SEX	1	6661.58	311.96	<.0001
RP×SEX	2	161.74	3.82	0.0231
SEL×SEX	1	43.38	2.03	0.1545
RP×SEL×SEX	2	19.71	0.46	0.6305
VIAL[RP,SEL], Random	12	85.47	–	**
BLOCK, Random	1	2208.61	–	**
ERROR	832	17719.37		

This reduction in immune function of SS males was not associated with an overall increase in the level of courtship: the analysis of the sexual behavior of C versus SS males with five females found no significant difference between them in their overall sex-

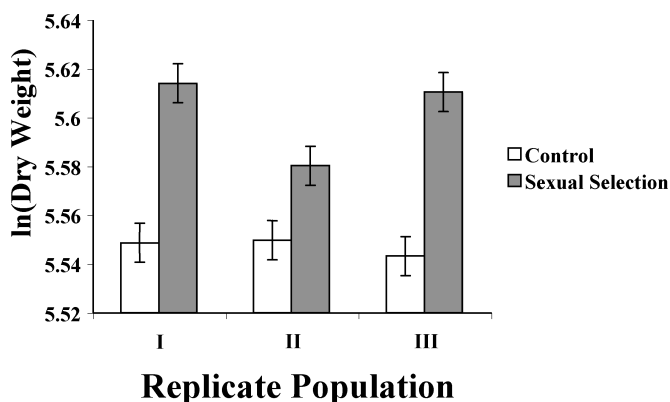


Figure 3. Dry weight of sexually selected and control adult males in ln(μg) (± SE). Females are larger but show a qualitatively similar pattern (Table 3).

ual activity (Table 5). However, further analysis showed that there was a highly significant difference in the timing of courtship activity between the two types of males. The SS males showed more sexual activity when they were placed in a fresh vial (measured on the second day in the vial). By the third day, the sexual activity of C and SS males declined, but the level of activity dropped much more in SS males ($P < 0.001$, see Contrast 2, Table 5; Fig. 5). Our analysis showed that this effect was not due to an aging effect of the flies, because both types of male showed the same overall level of courtship across both the first and second vial ($P > 0.75$, Contrast 1).

Selection response: sex differences in immune function

Under environmental conditions optimal for the expression of immune function of both sexes (excess food for females, absence of females for males; McKean and Nunney 2005), both males and females from sexually selected lines showed reduced immune function compared to controls (SELECTION, $P = 0.01$, Table 6A; Fig. 6). This difference could not be explained by size differences between sexually selected and control lines (Table 6B). We found

Table 4. Analysis of immune function (measured inversely by the number of *E. coli* remaining) in control (C) and sexually selected (SS) males. Main effects: SEL: C or SS; RPD: populations I, II, III including between-day differences; FEM: males in vials alone or with five females. *Escherichia coli* counts were log transformed to meet the assumptions of ANOVA. In (B) and (C), the interactions with the covariate were not significant. Significance levels of $P < 0.05$ are bolded.

Dependent variable: $\ln(E. coli)$				
Source	df	SS	F	P
(A) All main effects—response per male				
SELECTION (SEL)	1	6.405	16.92	0.0002
REPOPDAY (RPD)	2	17.959	–	**
RPD × SEL	2	0.756	1.00	0.3770
FEMALES (FEM)	1	56.309	148.83	<.0001
RPD × FEM	2	0.230	0.30	0.7389
SEL × FEM	1	0.250	0.66	0.4159
RPD × SEL × FEM	2	0.618	0.82	0.4427
VIAL[RPD,SEL], Random	42	5.270	–	**
ERROR	408	154.375		
(B) All main effects—average response per rearing vial. Covariate: mean male weight per vial.				
SELECTION (SEL)	1	0.596	7.08	0.0093
REPOPDAY (RPD)	2	5.138	–	**
RPD × SEL	2	0.163	0.97	0.3831
FEMALES (FEM)	1	11.795	140.21	<.0001
RPD × FEM	2	0.045	0.27	0.7662
SEL × FEM	1	0.040	0.48	0.4921
RPD × SEL × FEM	2	0.115	0.68	0.5082
LN(WEIGHT)	1	0.108	1.29	0.2594
ERROR	83	6.983		
(C) Main effects SEL and RPD—response per male housed with five females. Covariate: level of courtship.				
SELECTION (SEL)	1	4.489	15.79	0.0003
REPOPDAY (RPD)	2	11.371	–	**
RPD × SEL	2	0.058	1.03	0.3665
COURTSHIP	1	1.531	5.38	0.0214
VIAL[RPD,SEL], Random	42	4.656	–	**
ERROR	184	52.301		

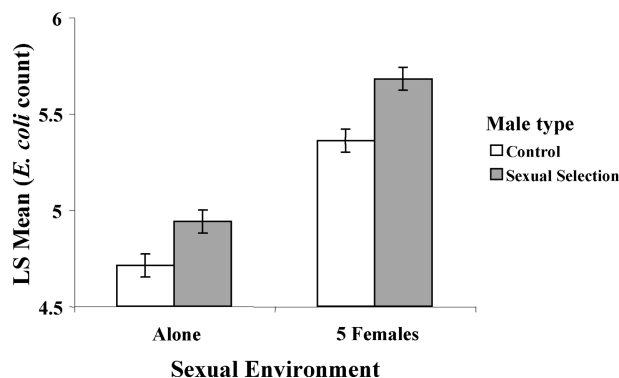


Figure 4. Antimicrobial immune function of sexually selected and control males housed in vials alone or with five females. High scores indicate poor microbial clearance, and are the least square means of $\ln(E. coli)$ remaining (\pm SE) from the analysis.

no evidence of sexual dimorphism in immune function, or that sexual selection caused a change in the pattern of immunological sexual dimorphism, because both SEX and SEX × SELECTION were nonsignificant (Table 6A).

Discussion

In this study we increased sexual selection on male *D. melanogaster* for 58 generations by artificially maintaining a male-biased sex ratio. The sexual selection had the predicted evolutionary effect of increasing the sexual competitiveness of sexually selected (SS) males relative to control (C) males (Fig. 2; Table 2). We used this experimental evolution to test three predictions of hypotheses concerning the immunological consequences of sexual selection. First, we showed that increased sexual

Table 5. Analysis of the courtship behavior of control and sexually selected males. Courtship was sampled on two days in vial 1 (days 3 and 4 of the experiment) and two days in vial 2 (days 6 and 7). The full model tests for differences in courtship rates summed over the four days of observations. In the repeated measures ANOVA, three contrasts tested for differences due to (1) fly age (comparing the mean courtship seen in vial 1 versus vial 2), (2) vial age (comparing the mean courtship seen on the first day in each vial versus the second day), and (3) the fly age \times vial age interaction (comparing the combined means of courtship of the first day in vial 1 and the second day of vial 2 to the second day in vial 1 with the first day in vial 2). The dependent variable was the angular transformation of the proportion of times that a male was observed courting per day. Significance levels of $P < 0.05$ are bolded.

	<i>F</i>	<i>P</i>
Full Model: Sum Courtship across all 4 days		
SELECTION (SEL)	1.31	0.254
REPPOPDAY (RPD)	11.99	<.0001
RPD \times SEL	2.67	0.072
VIAL(RPD,SEL)	0.90	0.645
Repeated Measures ANOVA: Daily		
Courtship Contrast 1: Fly Age		
SEL	0.001	0.978
RPD	116.93	<.0001
RPD \times SEL	2.67	0.072
VIAL (RPD,SEL)	0.97	0.537
Contrast 2: Vial Age		
SEL	13.53	0.0003
RPD	0.90	0.407
RPD \times SEL	1.06	0.350
VIAL (RPD,SEL)	0.83	0.757
Contrast 3: Fly Age \times Vial Age		
SEL	0.001	0.994
RPD	8.03	<.0001
RPD \times SEL	1.31	0.273
VIAL (RPD,SEL)	0.93	0.597

selection lowers immune function (Fig. 4; Table 4) demonstrating an evolutionary trade-off between sexual competitiveness and immune function. Second, even though sexual selection increased for males only, the evolutionary immunological consequences were independent of sex (Fig. 6; Table 6), contrary to the prediction that sexual selection promotes the evolution of immunological sexual dimorphism. Third, we found no evidence of a reduction in the condition-dependent immunological cost of increased mating effort in these sexually selected lines, contrary to the prediction of the ICHH (Fig. 4; Table 4). We also demonstrated, using the unselected base population, that flies successful in a competitive mating experiment exhibited reduced immune function relative to unsuccessful males, and that the immunological cost of successful flies relative to unsuccessful flies was increased by the presence of females, again contrary to the prediction of ICHH.

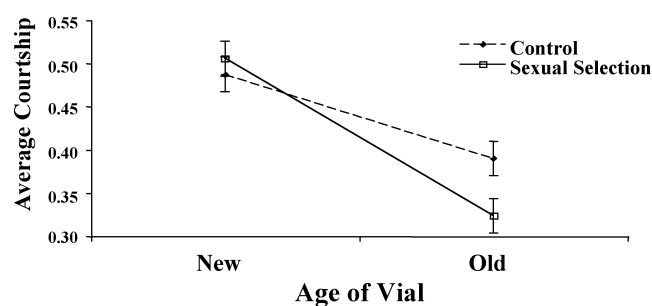


Figure 5. Changes in the courtship activity (average proportion of times a male was observed courting \pm SE) of sexually selected and control males in a new vial (the first day in a vial) and an old vial (the second day in a vial). Data were pooled across the two sequential vials (sampled on days 3 and 4 and days 6 and 7, respectively).

EVOLUTIONARY CHANGES IN MALE SEXUAL COMPETITIVENESS

In direct mating competition, sexually selected (SS) males showed increased sexual competitiveness compared to control (C) males.

Table 6. Analysis of sex differences in the number of *E. coli* remaining in control and sexually selected flies housed alone in vials supplied with excess dietary yeast. *Escherichia coli* counts were natural-log transformed to meet the assumptions of ANOVA. Significance levels of $P < 0.05$ are bolded.

Dependent variable: $\ln(E. coli \text{ count})$				
Source	df	SS	<i>F</i>	<i>P</i>
(A) ANOVA on individual values of <i>E. coli</i> counts.				
SELECTION (SEL)	1	4.624	7.300	0.012
REPPOP (RP)	2	7.616	–	**
RP \times SEL	2	0.869	0.686	0.512
SEX	1	1.112	1.753	0.186
RP \times SEX	2	0.741	0.585	0.558
SEL \times SEX	1	0.162	0.256	0.613
RP \times SEL \times SEX	2	0.644	0.508	0.602
DAY, Random	2	7.365	–	**
VIAL(RP,SEL), Random	28	4.959	–	n.s.
ERROR	275	174.375		
(B) ANCOVA using the rearing vial means for each sex of <i>E. coli</i> counts and dry weight.				
SELECTION (SEL)	1	1.111	4.821	0.030
REPPOP (RP)	2	1.794	–	**
RP \times SEL	2	0.329	0.715	0.494
SEX	1	0.131	0.569	0.454
RP \times SEX	2	0.264	0.573	0.567
SEL \times SEX	1	0.018	0.077	0.783
RP \times SEL \times SEX	2	0.251	0.544	0.583
LN(WEIGHT)	1	0.207	0.898	0.347
DAY, Random	2	0.923	–	n.s.
ERROR	57	13.130		

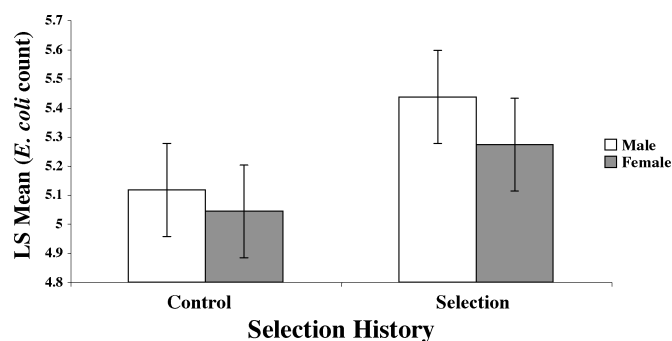


Figure 6. Sex differences in antimicrobial immune function of sexually selected and control flies. Shown are least square means of $\ln(\text{bacteria remaining}) \pm \text{SE}$ from the analysis.

This result was independent of whether males were tested with SS or C females. Although the qualitative pattern was consistent across the three replicate populations, the quantitative response to selection differed, with replicate population I showing a smaller difference than replicates II and III (Table 2, Fig. 2).

It is likely that at least some of the increased sexual competitiveness was due to the increased size of SS males who were on average 5% larger, by dry weight, than C males (Fig. 3). The evolved increase in body size resulted in a 3% increase in development time in SS flies (Table 3), revealing a genetic trade-off previously demonstrated (Nunney 1996). Similar evolutionary changes in body size have been reported in lines in which the opportunity for sexual selection was reduced: males and females from monogamous lines were smaller than flies from promiscuous lines (Promislow et al. 1998; Holland and Rice 1999; Pitnick et al. 2001; Rice and Holland 2005). However, in a study implementing experimental sexual selection in a design similar to ours there was no difference in the size of sexually selected flies and their controls (Wigby and Chapman 2004). In that study flies were kept in population cages with either 50 males and 50 females (equivalent to our control lines) or 75 males and 25 females (equivalent to our sexually selected lines). Assuming lottery polygyny (Nunney 1993), the effective population sizes of these two treatments are $N_e = 67$ and $N_e = 43$, respectively (compared to $N_e = 267$ and 256 in our control and selection lines), with remating slightly increasing these values depending on sperm precedence. The low effective population sizes in these lines (especially in lines in which the opportunity for sexual selection was increased) may have limited the evolutionary response to selection. Alternatively, the difference could be due to differences in the base populations used to initiate the experiments.

Although the advantage of larger males in competition for mates in *D. melanogaster* is well established (Partridge et al. 1987a, b; Wilkinson 1987; Markow 1988; Bangham et al. 2002) the observation that success in mating competition in the base population was not related to size ($P > 0.5$, Table 1) suggests that

the increased size of sexually selected males does not fully explain their increased sexual competitiveness. One possible factor is increased courtship. We established that the competitive advantage of SS males was not due to an overall increase in their courtship activity (Table 5), but that it was perhaps facilitated by their increased courtship when first entering a new bottle (Fig. 5). Such a behavioral trade-off (high courtship when first in a new bottle, lower courtship later) could be adaptive given the conditions of the selection experiment. Males would benefit from increased sexual activity immediately after transfer into a new bottle, due to last-male sperm precedence combined with the limited time available for egg laying. Other traits that could contribute to the increased sexual competitiveness of SS males include courtship song (Shorey 1962) and cuticular hydrocarbons (Ferveur 2005). However, the extent of within population variation in these traits, and its contribution to variation in mating success, has not been extensively studied.

IS THERE A TRADE-OFF BETWEEN SEXUAL COMPETITIVENESS AND IMMUNE FUNCTION?

Phenotypic correlation

Using unselected flies from the base population, we grouped males as “initially successful” (IS) or initially unsuccessful (IU) based on how rapidly they mated with virgin females under competitive mass-mating conditions. The subsequent manipulation of the sexual environment of these males, by placing them in vials either alone or with five females, confirmed a previously reported trade-off between male sexual activity and immune function (McKean and Nunney 2001, 2005). Overall our analysis indicated that more bacteria were recovered from IS than IU males, indicating reduced immunocompetence, an effect independent of male size (Table 1, Fig. 1).

The experimental manipulation of male sexual activity seemed to have a greater impact on IS males than IU males (see Fig. 1). Indeed, using independent contrasts, the immune function of IS males was significantly less than IU males when the males were tested after being housed with five females ($P < 0.05$), but not when tested after being alone ($P > 0.5$). Although this difference in the magnitude of the response was not great enough to promote a significant interaction, it does suggest that reduced immunocompetence of IS males is at least in part condition dependent.

These results support the trade-off hypothesis: that the mating success of IS males was, at least in part, due to redirecting investment away from the immune system. However, it is unclear what actual traits contributed to the increased initial mating success and the subsequent reduction in immune function. We have previously shown that the decline in immune function due to increased sexual activity is due to courtship, and not mating (McKean and Nunney 2001), an effect due in part to an apparent trade-off between

foraging and sexual activity (McKean and Nunney 2005). Although in this study we did observe reduced immune function in sexually active males (with five females) compared to those kept alone, there was no difference in the courtship and mating activity of IS and IU males (Table 1), although it should be noted that these behaviors were recorded several days after the competitive mating experiment.

One potential explanation of the lowered immune function of IS males was that all IS males (unlike IU males) were guaranteed to have mated at least once (during the initial mating trial). However, there was no difference in the number of bacteria recovered from IU males that had subsequently been observed mating versus IU males that were never observed mating. This result is consistent with the conclusion of McKean and Nunney (2001) that it is increased courtship, and not increased mating, that contributes to reduced immune function of males in vials with multiple females, and suggests that the drop in the immune function of IS males was not due to their single additional mating.

The finding of reduced immune function in successful (IS) males contrasts with a number of reports of positive phenotypic correlations between immune phenotypes and the expression of secondary sexual traits or mating success in a number of other arthropods, including in damselflies (Rantala et al. 2000; Siva-Jothy 2000), crickets (Ryder and Siva-Jothy 2000; Rantala and Kortet 2003; Rantala et al. 2004; Simmons et al. 2005), mealworm beetles (Rantala et al. 2002, 2003), horned beetles (Pomfret and Knell 2006), and wolf spiders (Ahtiainen et al. 2004). The observation of these positive phenotypic correlations has been taken as evidence in support of the ICHH. However, results demonstrating a positive phenotypic correlation between male sexual competitiveness (or the development of a secondary sexual characteristic) should not be viewed as supporting ICHH unless it can be definitively shown that the correlation arises as a consequence of underlying genetic variation (Kotiaho 2001).

Positive phenotypic correlation between fitness traits that show an allocation trade-off may occur if variation in the acquisition of resources (i.e., condition) is greater than the variation in the allocation of those resources (Van Noordwijk and Dejong 1986; Houle 1991; Reznick et al. 2000). Such variation can arise as a consequence of both environmental and genetic variation, and it is often the case that environmental variation can affect the magnitude and sign of phenotypic correlations when underlying genetic correlations are negative (Roff 2002). This makes the interpretation of data from studies of field-caught individuals problematical, because environmental variation is uncontrolled. Even in studies conducted under controlled laboratory conditions there may be hidden sources of environmental variation. For example, Nunney (1996) found significant between-vial variation in fly weight, and, in the present study, among-vial variation in wing length was close to significance ($F_{3,137} = 2.459$, $P = 0.065$). In both exam-

ples, the vials were established with equal densities of individuals under conditions of excess food. To avoid this problem, the IS and IU males in each replicate were from the same rearing bottle, reducing environmental variance affecting condition, and hence minimizing environmentally driven positive correlations. Under these conditions, we found no support for ICHH.

Genetic correlation

Our results, showing that sexually selected males have a higher mating success but lower immune function than controls (Fig. 3), support the hypothesis of a genetically based trade-off between sexual competitiveness and immune function. Regardless of the sexual environment, SS males cleared significantly fewer bacteria than did C males. Similar results demonstrating negative genetic correlations between various measures of immune function and sexually selected traits have been previously reported. In yellow dung flies the experimental removal of sexual selection for 12 generations resulted in a decline in sexual competitiveness and an associated increase in phenoloxidase activity (Hosken 2001). In the cricket *Teleogryllus oceanicus*, there is a genetic trade-off between sperm viability and antimicrobial, lysozyme-like activity (Simmons and Roberts 2005). Selection for improved antibody responses in chickens resulted in a correlated decrease in comb size, a sexually selected trait (Verhulst et al. 1999). However, selection for increased resistance to the parasitoid wasp *Asobara tabida* (Kraaijeveld and Godfray 1997) in *D. melanogaster*, while exposing a trade-off with larval competitive ability, resulted in a correlated increase in male mating success (Roff and Kraaijeveld 2003). It remains to be tested whether positive genetic correlation between parasitoid resistance and male mating success is real or arose as an unintended consequence of the selection procedure (Roff and Kraaijeveld 2003).

The mechanism of SS male superiority in mating competition with the C males is not known, but, as noted above, size was probably a factor. The SS males were on average larger than C males; however, the available evidence argues against size determining immune function. First, despite substantial size dimorphism there is no sex difference in immunocompetence (Table 6; see also McKean and Nunney 2005); second, within each sex, the immunological clearance rate of *E. coli* is independent of fly size (McKean and Nunney 2005); and third, the inclusion of dry weight into the statistical model did not change the significance of the main effect of selection (Tables 4B and 6A).

Genetic versus phenotypic correlation

There was a remarkable consistency in the patterns of phenotypic and genetic correlation, with both types of studies revealing a trade-off between sexual competitiveness and immune function. Furthermore, in both cases, reduced immune function does not seem to be due to an overall increase in courtship activity of more

successful males, although courtship has previously been recognized as a contributing factor to the phenotypic trade-off (McKean and Nunney 2001). However, in the SS flies, there was a significant shift in the timing of courtship, with higher levels of courtship (relative to control flies) soon after transfer to a new environment and lower levels later (Fig. 5). This courtship pattern was consistent in the sexually selected (SS) males across the three selected populations, raising the possibility that this courtship pattern is costly and drives the immunosuppression.

DOES SEXUAL SELECTION PROMOTE SEXUAL DIMORPHISM IN IMMUNE FUNCTION?

Sex differences in resistance to parasites and pathogens, and immune function are common in most animal species surveyed and even in dioecious plants (Zuk 1990; Zuk and McKean 1996). On average, males are more susceptible to infectious disease than females, an effect due in part to reduced male immune function compared to females (Zuk 1990; Zuk and McKean 1996). Ultimately these differences are thought to arise because of differences in how the sexes maximize their fitness (Zuk 1990; Zuk and McKean 1996; Rolff 2002; McKean and Nunney 2005). In particular, sex differences in immune function are predicted to be exaggerated as the mating system deviates from monogamy (Zuk 1990; Zuk and McKean 1996). The predicted relationship between the mating system and immune function may be complicated by sex-specific phenotypic plasticity (McKean and Nunney 2005) or if male and female life histories differ independent of male mating effort (Rolff 2002).

To the extent that sexual selection acts on genetic variation in males that is independent of females (i.e., on genes with sex-specific expression), we would expect that the immunological consequences of such selection to also be sex-specific, thus promoting changes in the sex difference in immune function. However, our results are inconsistent with the hypothesis that sexual selection promotes the evolution of immunological sexual dimorphism (Zuk 1990; Zuk and McKean 1996; Rolff 2002). In *D. melanogaster*, when each sex is tested under immunologically benign conditions (females with excess food; males in isolation), there is no sexual dimorphism for immune function (McKean and Nunney 2005). Increased sexual selection in males promoted a decline in both male and female immune function, but no dimorphism developed (Fig. 6). Similar positive intersexual genetic correlations for immune function have been reported in the yellow dung fly and in the mealworm beetle *Tenebrio molitor* (Rolff et al. 2005). These strong, positive intersexual genetic correlations could be an important source of sexually antagonistic ontogenetic conflict (Chippindale et al. 2001; Rice and Chippindale 2001) resulting in both males and females being unable to reach their sex-specific optima for immune function.

DOES SEXUAL SELECTION CHANGE THE CONDITION-DEPENDENT EXPRESSION OF IMMUNITY?

The ICHH relies upon two crucial assumptions. First, it is assumed that a limited pool of resources is allocated between the competing demands of sexual competitiveness and immune function. Second, defining the level of this pool of resources as condition (Rowe and Houle 1996), it is assumed that there is genetic variation for condition. Depending on the extent of the genetic variation for condition, there are two alternative outcomes relating the expression of immunocompetence and sexual competitiveness in a population experiencing an increase in the intensity of sexual selection. If genetic variation for condition is greater than the genetic variation in the allocation of resources between immune function and mating effort, this would promote a positive genetic correlation among fitness traits even though there are underlying patterns of competitive allocation of resources (Houle 1991; Reznick et al. 2000). Under such conditions, we would expect selection to favor genetic variants promoting an increase in the pool of resources that can be allocated to both sexual activity and immune function (see de Jong and van Noordwijk 1992). Thus, the observed trade-off between male sexual competitiveness and immune function indicates that high levels of additive genetic variation for condition affecting the joint expression of these were not present in our population of *D. melanogaster*.

Alternatively, given genetic variation for condition appeared to be less than genetic variation for allocation (leading to the observed trade-off), ICHH could exploit genetic variation for condition dependence (Rowe and Houle 1996). Such "efficiency" variation would be revealed if favored genotypes pay a lower marginal immunological cost for a given increase in mating effort (Rowe and Houle 1996; Lorch et al. 2003). Thus, in the context of the experimental sexual selection presented here, we would expect males from the sexually selected population to be less immunocompromised (relative to their solitary baseline) when their sexual activity increased.

We tested this second prediction by comparing immune function in SS and C males across two environments previously shown to have dramatic effects on male condition, the presence or absence of females (McKean and Nunney 2005). We found no evidence of any change in the condition-dependent immunological costs in males from sexually selected lines (SELECTION \times NUMBER OF FEMALES interaction, $P = 0.42$, Table 4A, Fig. 4). There was also evidence that SS males tended to pay a higher (rather than the predicted lower) marginal cost for a given level of courtship. The average courtship levels did not differ between C and SS males (Table 5), but there was a significant negative phenotypic correlation between courtship levels and immune function within the treatment groups (Table 4C).

Although we have addressed key issues of the ICHH, our experiment is still not a complete test. It could be that if the sexual

selection had been carried out in an environment containing parasites and pathogens, the pattern of evolutionary change suggested by the ICHH could contribute to either more rapid evolution of resistance to a particular pathogen, or could accelerate coevolutionary cycles between pathogen and host.

Since its original publication in 1992 (Folstad and Karter 1992) the ICHH has received a great deal of attention, with a recent review noting that it is one of the top 10 cited papers in evolutionary ecology in the last 10 years (Roberts et al. 2004). Despite this attention, a major assumption of the ICHH, that there is genetic variation in the immunological costs associated with the effort ensuring mating success, had not been previously tested (Kotiaho 2001).

Summary

Our results clearly show that when sexual selection is intense, mating success comes at an immunological cost. This cost is shared between males and females, resulting in no change in immunological sexual dimorphism. This intersexual genetic correlation could be an important source of ontogenetic conflict between the sexes, resulting in neither sex reaching its sex-specific optima for immune function. However, we found no evidence supporting the ICHH in this insect population. Although there was a genetic trade-off between male immune function and sexual competitiveness, both the phenotypic and genetic correlations between competitiveness and immune function were negative and we found no evidence that sexual selection acted to change the pattern of condition-dependent expression of immunocompetence.

These results are consistent with the general conclusion that sexual selection is a significant evolutionary factor shaping patterns of immunocompetence (Zuk and Stoehr 2002; Schmid-Hempel 2003, 2005). In particular, the cost of increased disease susceptibility could be important in limiting increases in sexual competitiveness in populations experiencing intense sexual selection. Such costs may be exaggerated when the level of sexual selection changes, because a high intersex correlation will delay (or even prevent) males in reaching their sexually selected optimum, and put females at increased risk of disease.

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