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Phillip G. Byrne
University of Wollongong, pbyrne@uow.edu.au

W R Rice

G R Rice

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Abstract
The Drosophila melanogaster laboratory model has been used extensively in studies of sexual conflict because during the process of courtship and mating, males impose several costs upon females (e.g., reduced fecundity). One important difference between the laboratory and the wild is that females in the laboratory lack a spatial refuge from persistent male courtship. Here, we describe two experiments that examine the potential consequences of a spatial refuge for females. In the first experiment, we examined the influence of a spatial refuge on mating rate of females, and in the second one we examined its influence on females' lifetime fecundity. We found that females mated about 25% less often when a spatial refuge was available, but that the absence of a spatial refuge did not substantially increase the level of male-induced harm to females (i.e., sexual conflict).

Keywords
persistent, environment, refuge, laboratory, effect, drosophila, courtship, male

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Effect of a refuge from persistent male courtship in the *Drosophila* laboratory environment

Phillip G. Byrne,*,† Gavin R. Rice* and William R. Rice[1,*]

*Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, California, USA;
†School of Botany and Zoology, Australian National University, Canberra, Australia

Synopsis The *Drosophila melanogaster* laboratory model has been used extensively in studies of sexual conflict because during the process of courtship and mating, males impose several costs upon females (e.g., reduced fecundity). One important difference between the laboratory and the wild is that females in the laboratory lack a spatial refuge from persistent male courtship. Here, we describe two experiments that examine the potential consequences of a spatial refuge for females. In the first experiment, we examined the influence of a spatial refuge on mating rate of females, and in the second one we examined its influence on females' lifetime fecundity. We found that females mated about 25% less often when a spatial refuge was available, but that the absence of a spatial refuge did not substantially increase the level of male-induced harm to females (i.e., sexual conflict).

Introduction

The use of laboratory studies to assess evolutionary processes that occur in the wild requires careful interpretation. When organisms that are adapted to wild conditions are assayed in a novel laboratory environment, many artifacts are possible owing to the mismatch between past selection on the organisms and the demands of the novel environment (Service and Rose 1985, Sgrò and Partridge 2000). Similarly, when highly inbred laboratory strains of organisms are studied, many of the traits they exhibit may have evolved despite natural selection during adaptation to the laboratory environment (rather than because of it), due to drift overpowering selection in populations with small effective size. One approach that attempts to circumvent these difficulties is "laboratory island analysis" (Rice et al. 2005, 2006) in which large outbred populations are permitted to adapt to a competitive laboratory environment for hundreds of generations. In this case, it is still inappropriate to directly extrapolate results observed in the laboratory environment to nature, but it is possible to derive evolutionary principles from studies of these microcosms, and then use those principles to extrapolate back to wild conditions. However, if the laboratory environment has characteristics that qualitatively change the functional form of selection compared to that experienced by at least some populations in the wild, then some principles derived from evolutionary island analysis may have no application to natural populations.

The *Drosophila melanogaster* laboratory model has been used extensively in the empirical assessment of sexual conflict (Partridge et al. 1987; Fowler and Partridge 1989; Chapman et al. 1993, 1995; Rice 1996; Holland and Rice 1998; Civetta and Clark 2000; Prout and Clark 2000; Sawby and Hughes 2001; Pitnick and García-González 2002; Orteiza et al. 2005; Stewart et al. 2005). One important difference between nature and the laboratory is that females in nature can leave high-quality feeding and oviposition sites, and thereby avoid persistent courtship from males that aggregate at these sites. In the laboratory, females cannot utilize such a spatial refuge and, therefore, sexual conflict there may be far more severe than in nature. For this reason, the conflict observed in the laboratory may be irrelevant to natural populations of *Drosophila*. However, although spatial refuges are available in nature, to use them females must give up access to high-quality resources, and this trade-off may largely preclude their use of spatial refuges.

Methods

In this study, we carried out two experiments to evaluate the importance of lack of a spatial refuge in the laboratory with respect to measures of

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[1]E-mail: rice@lifesci.ucsb.edu
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male-induced harm to females. In the first experiment, we focused on female remating rate with, and without, a spatial refuge and determined whether or not females remated less frequently when provided with a spatial refuge from males. There is selection against remating in our base population of flies (Linder and Rice 2005; Kuiper et al. 2006; Lew et al. 2006), so remating rate was a useful measure of costs to females. In the second experiment, we focused on female lifetime fecundity with, and without, a spatial refuge. A refuge was made by attaching an extension to each vial that provided both visual and spatial isolation from the main group of males (that congregated near a single source of food) and thereby permitted at least partial escape from persistent male courtship.

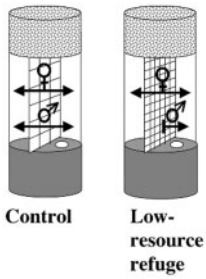

Fig. 1 Diagram of the experimental vials used in the remating experiments. They were divided into two chambers by a mesh screen, bordered at the base by culture medium and at the top by a rayon plug. Females were nutritionally manipulated to be small, and males to be large, so that females, but not males, could pass through the dividing screen. Live yeast (white ellipse) was placed in the chamber with males. In the control, larger mesh permitted both sexes to move freely between chambers.

Cultures

Flies were used from two laboratory populations (LH_M) and (LH_M-*bw*). LH_M is a large (number of eggs used to start each generation is >9000 and number of breeding adults ≈1800), moderate-density outbred population originally derived from 400 mated females collected by Larry Harshman in 1991 from an orchard in central California. For over 320 generations before the start of these experiments, the LH_M population had adapted to laboratory culture (for culturing details see Rice et al. 2005, 2006). Briefly, during the juvenile and early adult stages (Days 1–12 of the 2-week generation cycle) the flies were dispersed among 56 "juvenile competition vials" at a density of 150–200 individuals per vial. On Day 12, when most individuals had fully matured, flies were mixed among vials, culled to 16 pairs per vial and transferred to 56 "adult competition vials" in which males competed to fertilize females and females competed for a limiting resource (10 mg live yeast applied to the surface of 10 ml of cornmeal/molasses medium containing killed-yeast) that has a strong positive effect on their fecundity (Linder and Rice 2005). Eighteen hours before the end of the 2-week generation cycle, the flies were transferred to un-yeasted "oviposition vials." Eggs laid during this time were pared down to 150–200 per vial and used to begin the next generation. Because only eggs laid in the oviposition vials were used to begin the subsequent generations, egg production during this 18 h period constitutes a female's lifetime fecundity.

The LH_M stock expresses the wild-type red-eye color. The LH_M-*bw* population is a replica of the LH_M population that carries a brown-eyed (*bw*) recessive marker that has been introgressed through 13 backcross generations into the LH_M genetic background. Both populations were cultured under a photoperiod of 12 h light: 12 h darks at 25°C.

Remating experiments

Remating trials were staged in culture vials that were divided into two chambers by a mesh screen (Fig. 1). These "remating" vials closely matched the "adult competition" phase of the normal propagation of the LH_M base population (see above). By manipulating their larval density (see below), we reduced the body size of females so they could cross the mesh, and move freely between both sides of the chamber. The small adult body size used in these experiments was observed in our base population as part of the normal variation in this phenotype. In contrast, we made the body size of males consistently large (see below) so they could not move through the mesh, subsequently restricting them to one chamber. Two experimental treatments were established. In the low-resource refuge treatment (Fig. 1, right), once-mated females were introduced into the chamber that contained no live yeast and no males. The other chamber contained both live yeast and males. In that one, high-quality resources (live yeast) were coupled with persistent male courtship but a lower-quality, male-free refuge was present. In the no-refuge treatment (Fig. 1, left), we used a larger-diameter mesh that permitted unrestricted access of both sexes to both chambers, one of which contained live yeast.

Females could potentially remate over a 48 h period during an experimental trial, the normal duration in the "adult competition" phase of the life cycle. In both experimental treatments, the body size of females was reduced. We ran four replicates of the experiment over 4 consecutive days (blocks), and the

means for vials were used for statistical analysis. After treatment, females were allowed to lay eggs for 48 h and their progeny were reared to maturity. Using different eye-color markers for primary and subsequent males, we were able to score whether females remated, or not, based on the eye colour of their progeny. Experimental females were homozygous for the recessive *bw* marker (*bw/bw*) and their primary mates (bw^+/bw^+) were red-eyed and secondary mates (*bw/bw*) were brown-eyed (see below). Therefore, if a female did not remate, all her progeny (bw^+/bw) would have red eyes due to the bw^+ allele contributed by the red-eyed (bw^+/bw^+) male from the primary mating. If a female remated, however, then some, and usually the majority, of her progeny would have brown eyes (*bw/bw*) because secondary males were also homozygous for the *bw* marker (*bw/bw*), and in *D. melanogaster* the last male to mate typically gains sperm precedence.

Technique for controlling body size

To alter the body size of experimental flies, we manipulated larval density. The normal density of the flies in the LH_M population is 150–200 fertilized eggs per 10 dram culture vial containing ~10 ml of medium. To generate females of small body size, 100 eggs were randomly sampled from the LH_M population and placed in a 10 dram vial containing only 1 ml of medium. Vials were incubated under standard culture conditions (25°C; 12 h light/12 h dark) and after 8 days virgin females were collected (within 6 h of eclosion) under brief CO_2 anesthesia. Females were stored in un-yeasted vials at 16/vial and allowed to mature for 3 days. Virginity was assured by checking vials for hatched eggs. The average body mass (±SE) of females (i.e., dry weight at 2 days of age, and prior to feeding on live yeast), estimated from a sub-sample of the experimental flies, was 0.188 mg ± 0.0020 ($n = 308$, normal body size is 0.263 mg, so body size was reduced by 28.5%). To generate males of consistently large body size (i.e., males that were within the normal range of body size, but with small body sizes excluded) 50 randomly sampled eggs were placed in a 10 dram vial ($n = 40$) containing 10ml of medium. Vials were incubated at 25°C and males were collected after 12 days (~3 days posteclosion). Males were collected under brief CO_2 anesthesia the night before experimentation and stored at 16/vial. The average body mass of males (dry weight ± SD) was estimated to be 0.250 ± 0.0353 mg ($n = 200$). Normal body size, based on a sample of 300 flies at normal density, was 0.248 ± 0.042 mg, so body size of males was only increased by 0.4%, but its variance was reduced by 70%, thereby eliminating the very small males that could pass through the mesh.

Insemination of virgins

Prior to experimental treatment we used LH_M males to inseminate LH_M-*bw* virgins. The LH_M males were produced using the standard protocol used to culture the LH_M base population and they were collected on Day-12 (~3 days posteclosion) of their 14 day culture cycle. On the morning of the experiment, we premated females by transferring, without anesthesia, 48 LH_M males into a culture vial containing 16 LH_M-*bw* virgin females. After 120 min, flies were sorted under brief CO_2 anaesthesia. Previous experiments in our laboratory demonstrated that females rarely mate more than once under this procedure (Rice 1996; Holland and Rice 1999). Females were returned to the culture vial and left to recover for 1 h before being randomly assigned to experimental treatments.

Insemination controls

To test how efficiently LH_M males inseminated LH_M-*bw* females, a series of matings were conducted in parallel with the main experiment. These control matings followed the same protocol as the experimental matings, except that after the 2 h exposure to males the females were individually placed in smaller culture tubes (containing medium and live yeast) and allowed to oviposit for 48 h. After 2 days, the presence of larvae determined successful insemination. Insemination rate averaged (±SE) 94.21 ± 1.44%.

Experimental protocol and data analysis

Following primary insemination, females were transferred, without anesthesia, from storage vials into one chamber of the partitioned experimental vials (Fig. 1), the other chamber contained the 16 LH_M-*bw* males. Males were transferred into experimental chambers just prior to female transfer. Four repeats (blocks) of the experiment were made over 4 consecutive days. On Day 1, we prepared two vials with a refuge and three no-refuge control vials. For each of the remaining 3 days, we made between 8 and 11 vials for the low-resource refuge treatment, and three no-refuge control vials. Independently derived flies of each sex were used for each block. Flies were left in the "remating" vials for 48 h, a duration corresponding to the foraging period for live yeast in the "adult competition" vials during the standard culture of the base LH_M population (see above). Following treatment, females were separated from males under light CO_2 anesthesia and placed

individually in test tubes (containing medium and live yeast), for 48 h of egg deposition. Females that produced broods with all red eyes had not remated and those that produced broods with some brown eyes had remated. Data points were vial proportions of remated females. To test for differences among experimental treatments we used 10 000 bootstraps of the data to calculate 95% Confidence intervals (CI) for the difference in mean mating rate between vials with and without a refuge (Resampling Statistics Excel add-in 2.0). In these bootstraps, the resampled data were the 13 means from the 13 no-refuge control vials and the 31 means from 31 refuge vials. Data from all four replicates were pooled during bootstrapping. Because our mating controls showed that ~6% of females were unmated prior to treatment, this value was subtracted from the original remating values (when expressed as averages in figures) to provide a more accurate estimate of remating rates.

Total male-harm experiments

Flies were collected from the stock population of LH_M at the time when flies were being transferred to the "adult competition" phase of their life cycle (see cultures above). At this time there is a large excess of adults, compared to the number needed to propagate the stock, and these extra flies were used in the experiment. Paired "adult competition" vials were constructed (16 males and 16 females per vial), with each pair placed side by side in the incubator to control for small differences in lighting, temperature, and other environmental variables that vary with position in an incubator. One vial was propagated using the normal protocol for the LH_M stock (see above) and the other was treated the same way except that a spatial refuge was attached to the vial (Fig. 2). The spatial refuge contained no food but it permitted escape of females from persistent male courtship by temporarily moving away from food where males congregated.

At the end of the 2 day "adult competition" phase of the life cycle, the females were placed in individual, unyeasted vials for the 18 h duration of the oviposition phase of the lifecycle, and then their total fecundity measured by counting the number of eggs deposited in these vials. Ten paired vials (i.e., a no-refuge vial and a low-resource refuge vial; Fig. 2) were assayed. In one block there were four paired vials and in the second block, processed 2 weeks later, there were six paired vials. After egg counts were made at the conclusion of the experiment, a total of 10 000 bootstraps of the data (10 differences: mean fecundity with a refuge present minus that when a refuge was absent) were used to calculate 95%CI for the difference in fecundity between vials with and without a refuge. Data from both replicates were pooled during bootstrapping. Lastly, scans of the female refuges were taken at approximate half-hour intervals during daylight hours. The number of males and females observed in the refuges was recorded at each scan to ascertain the proportion of flies using the refuge, and to ascertain any differences between the sexes in the use of the refuges.

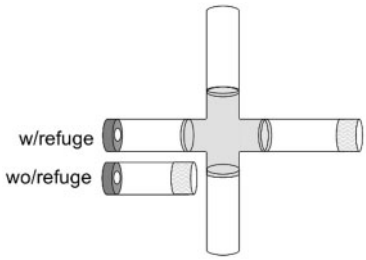

Fig. 2 Diagram of the experimental vials used in the experiments on total female fecundity. The culture medium is depicted by the dark column to the left of each vial, and the white ellipse on the surface of the culture medium depicts the live yeast. The no-refuge vial (lower-left) was a standard "adult competition" vial (see methods) and the low-resource-refuge vial (right and above) was identical except for the attached, T-shaped extension (affording spatial and visual isolation from the males that congregated around the food source).

Results

Remating experiments

Remating rate of females was higher when a refuge was absent (mean remate rate = 43.4 ± 4.2%) compared to when it was present (mean remate rate = 32.0 ± 3.2%) (Fig. 3). The difference (without refuge—with refuge) in mean remate rate was 11.2% and the bootstrap 95% CI for the difference was (1.4, 21.5). These data demonstrate that remating rate declined by about 25% in response to the presence of a spatial refuge from persistent male courtship.

Total male-harm experiments

During the first 4 h after the flies were introduced into the "adult competition" vials no flies were observed in the spatial refuges. At this time, the flies had just been moved from the depleted food in the "juvenile competition" vials to the new food in the "adult competition" vials, so all flies were probably actively feeding. For the remainder of Day-1, an average of 5.05 ± 1.50% of the flies utilized the refuge, and of these 87.07 ± 4.97% were females. On the second day, 19.12 ± 4.05% of the flies utilized

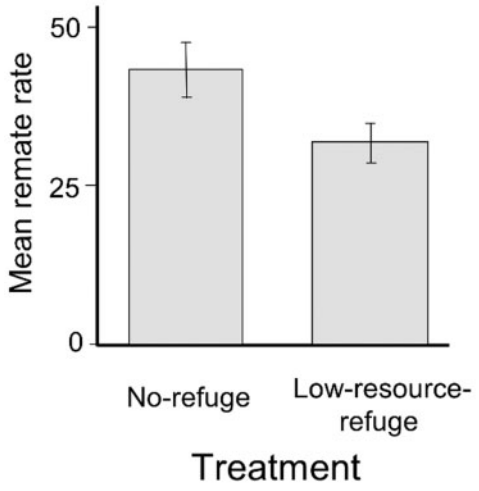

Fig. 3 Remating rate versus the experimental treatment. Remating rate of females (percent ± SE) is affected by the presence of a spatial refuge from persistent male courtship.

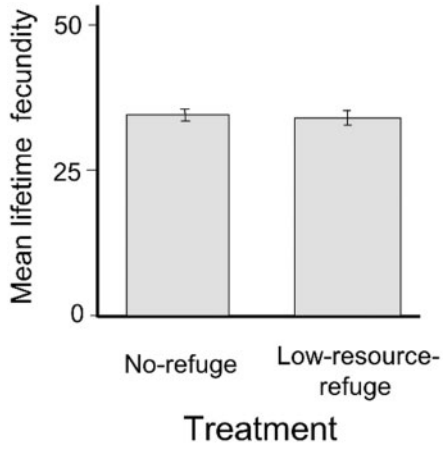

Fig. 4 Lifetime fecundity versus the experimental treatment. Female adult fitness (number eggs ± SE) is not affected by the presence of a spatial refuge from persistent male courtship.

the refuge and 66.97 ± 2.82% of them were females. Overall, females outnumbered males in the refuge by a factor of about six on Day 1 and two on Day 2.

The mean fecundity of females with (34.4 ± 1.13) and without a refuge (34.7 ± 0.82) was nearly identical (Fig. 4). The 95%CI for the difference (Δ) in mean fecundity with a refuge and without one overlapped zero and was narrow (−2.1,2.7), indicating that there was no measurable increase in female fecundity in response to the presence of a refuge, and that any undetected effect, owing to insufficient statistical power, was small (<2.5 eggs).

Discussion

Both the assay of remating rate and that of lifetime fecundity indicate that the presence of a spatial refuge does not markedly influence the level of male-induced harm to females in the laboratory. Remating is known to be harmful to females due to the influence of seminal fluid (Chapman et al. 1995, Kuiper et al. 2006), and previous studies in which newly mated females were reared in a male-free environment indicated that persistent male courtship and remating are harmful to females in our LH_M population (Linder and Rice 2005, Stewart et al. 2005), so we expected that the presence of a refuge might lead to a lower remating rate. The observed significant reduction in female remating when a refuge was present indicates that the lack of a refuge in the laboratory has an effect on male–female interactions, but the fact that remating rate was reduced by ~25% indicates that this effect is quantitative, rather than qualitatively changing the net harmful effect to females of persistent male courtship. The substantial level of remating that was observed, despite the presence of a spatial refuge, suggests that the male-induced harm to females observed in many past laboratory studies is not an artifact attributable to the absence of a spatial refuge in the laboratory. This interpretation is supported by our second experiment in which we assessed the influence of a spatial refuge on lifetime fecundity of females. We found no evidence for any substantial benefit to females when they were provided with a spatial refuge, despite the fact that females did use refuges and they experienced a substantially reduced operational sex ratio there. This second experiment indicates that although the presence of a spatial refuge did lead to reduced remating rate (experiment 1), it did not lead to an increase in female fitness, probably because of the trade-off between use of the refuge and the corresponding loss of access to high-quality feeding sites. Previous experiments have shown that in the laboratory environment lifetime fecundity of females is strongly influenced by the amount of live yeast that they secure during the "adult competition" phase of their life cycle (Linder and Rice 2005, Stewart et al. 2005). In the natural environment, the trade-off that we observed between remating rate and access to high-quality feeding sites may not be so closely counterbalancing, and the reduced remating rate experienced by females made possible by the availability of a refuge may have a net benefit.

A potential confound with our experiments on remating is that females were nutritionally manipulated to have small body size (and hence the males were relatively larger than normal), and this experimental treatment may have influenced decisions by females as to whether to remate. For example,

two recent studies reported that larger males harm females more than do smaller males (Pitnick and García-González 2002; Friberg and Arnqvist 2003). Another line of evidence that larger males may be more harmful to females is the observation that the long-term survival of females declines with the amount of seminal fluid that they received (Chapman et al. 1995). Therefore, the smaller females used in our remating experiments might be harmed, in the currency of lifetime fecundity, more by males than would normal-sized females. However, the disparity in body size used in our experiments on remating only makes our data conservative when evaluating the importance of a spatial refuge in terms of levels of male-induced harm in the laboratory.

These experiments indicate that while it is true that the laboratory environment does not provide the spatial refuge that females have available in nature, when such a refuge is provided, females are harmed as much as when they are absent. We observed that females did, in fact, use the spatial refuge to a far greater degree than did males, thereby benefitting from a substantially reduced operational sex ratio. However, at any single point in time the majority of females were not observed to be using the refuge, possibly because the benefit of avoiding persistent male courtship was more than offset by the benefit of feeding, despite the presence of males.

In summary, these experiments indicate that the lack of a spatial refuge in the laboratory is not a major factor limiting the use of the *D. melanogaster* model for the study of sexual conflict. It would be wrong to try and directly extrapolate findings from the laboratory to the field. However, in the context of laboratory island analysis, we think that the lack of a spatial refuge does not make the laboratory environment so different from nature that meaningful principles cannot be deduced from study of this microcosm.

Acknowledgments

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