



# Experience improves courtship in male fruit flies

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(Received 3 October 2003; initial acceptance 15 March 2004;  
final acceptance 11 August 2004; published online 25 March 2005; MS. number: A9713R)

Much of the research on learning in fruit flies has focused on the genetic and cellular basis of learning. The evolutionary relevance of learning in these tiny, short-lived insects is not well understood. Relying on the knowledge that male fruit flies learn in the context of courtship, I tested whether such learning improves male courtship. I found, first, that compared with inexperienced males, males with experience courting recently mated, unreceptive females were slower to begin courtship of novel recently mated females and spent less time courting such females. The experienced males, however, began courting virgin females sooner than did inexperienced males and courted such females as long as did inexperienced males. Second, compared with males experienced with recently mated females, males experienced with immature, unreceptive females were faster to approach either virgin or recently mated females and courted virgin females longer. These results indicate that courting experience permits male fruit flies to refine their courtship behaviour in a way that could increase their mating success.

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The long and controversial search for learning in fruit flies (*Drosophila melanogaster*) culminated with convincing demonstrations of significant learning by adults and larvae, and cleared the path for fruitful research on the genetic and cellular basis of learning, in which fruit flies have served as a prime model system (reviewed in: Quinn 1984; Tully 1996; Waddell & Quinn 2001; Dubnau et al. 2003). The adaptive significance of learning in fruit flies, however, has not been well documented. Exceptions include (1) work demonstrating that naïve mature males initially court recently eclosed, immature males, but quickly habituate to avoid them (Gailey et al. 1982, 1985), (2) research indicating associative learning by fruit fly larvae, enabling them to prefer odours associated with higher-quality food and to avoid odours linked to simulated predation (Dukas 1999), and (3) a recent finding that male *D. melanogaster* experienced with courting *D. simulans* females learn to decrease such interspecific courtship while maintaining normal levels of courtship towards conspecific females (Dukas 2004).

Neurogenetic studies on learning in fruit flies commonly use a 'courtship conditioning' protocol. Such studies indicate that, compared with inexperienced males, males experienced with unreceptive, fertilized females subsequently show reduced courtship towards anaesthetized virgin females (Siegel & Hall 1979; Tompkins et al. 1983;

McBride et al. 1999). Although courtship conditioning experiments elegantly demonstrated learning in the context of courtship, the results were puzzling because experience with mated females, which is very common in nature (Spieth & Ringo 1983), caused males to show partial avoidance of sexually receptive, virgin females. Perhaps the use of anaesthetized virgin females was the cause for the nonintuitive results. Using a different protocol, Reif et al. (2002) also documented that male *D. melanogaster* reduced courtship towards conspecific mated females over time, but Reif et al. did not test the males with virgin females.

Starting from the data on courtship conditioning, I evaluated the possibility that learning can improve courtship by male fruit flies. Specifically, I first asked whether, compared with inexperienced males, males with experience courting unreceptive, fertilized females would subsequently show a selective reduction in courtship towards fertilized females but not virgin females. In a follow-up experiment, I tested whether males experienced at courting unreceptive, immature females also would show a similar selective reduction in courting fertilized females but not virgin females.

## GENERAL METHODS

I used a stock of *D. melanogaster* flies initiated from wild flies collected in Vancouver, British Columbia in early summer 2001. The flies were kept in population cages (20 × 20 × 35 cm) containing a few thousand

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individuals. Each population cage contained two standard 240-ml food bottles each containing 50 ml of standard fly medium consisting of sucrose, corn meal, yeast, agar, benzoic acid, methyl paraben and propionic acid. The population cages were maintained in an environmental chamber at 25 °C, 70% RH on a 16:8 h light:dark cycle with lights on at noon.

All flies used for the experiment were developed at low densities of approximately 300 larvae per standard food bottle. Before collecting virgin flies at approximately 7 h posteclosion, I mixed flies from approximately 12 bottles in a single cage to randomize bottle effects. I then anaesthetized small numbers of flies with CO<sub>2</sub>, sexed them and placed groups of 10 males per vial and groups of 30 females per vial. I used standard 40-ml vials containing 5 ml of medium. The vials were housed in the environmental chamber for 4 days before the experiment, which was always conducted between 0800 and 1300 hours. All flies were used only once. All males were virgin with no experience with females after they were sexed and placed in the vials. The virgin females had no experience with males after sexing. Each female designated as 'mated' was placed in a vial with two males and observed to complete a normal mating approximately 24 h before the experiment. The immature females used in experiment 2 were less than 20 h posteclosion. Such females elicit intense courtship but typically do not mate (Manning 1967; Tompkins et al. 1983).

## EXPERIMENT 1: EXPERIENCE WITH MATED FEMALES VERSUS INEXPERIENCE WITH FEMALES

### Rationale

Experiments with naïve fruit flies (*D. melanogaster*) indicate that the males are typically indiscriminate and court both receptive and unreceptive conspecific and interspecific females (Spieth & Ringo 1983; Noor 1996; Dukas 2004). Female fruit flies show large geographical variation in pheromonal composition. For example, Jallon & David (1987) compared pheromonal composition of females from three *D. melanogaster* strains, Canton-S, Oregon and an Ivory Coast population. Proportions of the major female aphrodisiac, 7,11 heptacosadiene were 0.28, 0.197 and 0.028, respectively, and proportions of the major antiaphrodisiac, 7-tricosene, were 0.035, 0.05 and 0, respectively. Such variation might select for males that are initially indiscriminate but can learn through experience with females' pheromones and behaviour to focus on courting receptive females. For example, an inexperienced male might not know whether the first female he approaches is a recently mated and hence unreceptive female, a receptive virgin, or a distantly mated female ready to mate again. Females' pheromonal compositions and responses to males may provide the males with time- and place-specific information about the set of female pheromones and behaviours most likely to result in mating.

Based on this rationale, I predicted that (1) compared to inexperienced males, males experienced with unsuccessful

courtship of recently mated females would show selective reduction of courtship directed at recently mated females but not towards virgin females, and, (2) experienced males would be slower than inexperienced males at initiating courtship with recently mated females but not with virgin females.

### Methods

The experiment had four replicates conducted on different days, each consisting of 10 sessions, with each session including eight males. Hence, I tested a total of 320 males. The experimental protocol consisted of all four combinations of two male treatments (experienced and inexperienced) during the experience phase, and two female states (mated and virgin) presented during the successive test phase. All fly transfers during the experiment were done with gentle aspiration.

#### Experience phase

Each session, I randomly selected four males for the 'experienced' treatment and placed them individually into empty vials and then added two mated females to each of these vials. I also randomly selected four males for the 'inexperienced' treatment and placed them individually into empty vials. Frequent observations of the males assigned to the experienced treatment indicated that most of the males courted the mated females during the experience phase, but none mated. The experience phase lasted 60 min.

#### Test phase

When the experience phase had ended, I transferred each of the eight males into a fresh, empty vial. Then (1) two males of the experienced treatment each received two virgin females, (2) two males of the experienced treatment each received two mated females, (3) two males of the inexperienced treatment each received two virgin females, and (4) two males of the inexperienced treatment each received two mated females. The time between the experience phase and test phase was approximately 10 min. I placed two females with each male to reduce variation in courtship caused by interfemale variation.

The two assistants who conducted the observations were pretrained to eliminate interobserver differences and blind to male and female treatment. Each assistant received four randomly selected vials containing (1) an experienced male and two virgin females, (2) an experienced male and two mated females, (3) an inexperienced male and two virgin females, and (4) an inexperienced male and two mated females. The assistants recorded the start and end of each courtship activity, which included all 'following', 'wing vibration' and 'mounting attempts' (Cobb et al. 1985). I later calculated the total courtship duration for each male. Courtship duration has been widely used in studies on the effect of experience on male courtship (e.g. Siegel & Hall 1979; McBride et al. 1999) and is highly positively correlated with other measures such as the number of courtship bouts, wing vibrations and mounting attempts (Dukas & Mooers 2003). In addition

to recording courtship duration, the assistants also recorded the start of mating if it occurred. The test phase lasted 15 min.

### Analyses

The main statistical analysis was done on arcsine-transformed proportions of the time spent courting out of the total time available. In trials with no matings (99% of the trials with mated females and 24% of the trials with virgin females), the time available for courtship was 15 min. In trials with matings, the time available for courtship was the duration prior to mating.

I also examined the rank order of courtship latencies. Within each session, I ranked each of the eight males according to their courtship latency, such that the first male to court received a score of 1 and the last male to court received a score of 8. Males with tied scores each received the mean of their scores. Thus, in this analysis, lower numbers corresponded to shorter courtship latencies. I could not analyse the actual courtship latency because it was not recorded in this experiment. The statistical tests did not violate ANOVA assumptions.

### Results

Male experience significantly affected the proportion of time males spent courting in the test phase (Fig. 1). Inexperienced and experienced males spent similar proportions of time courting virgin females. Compared with inexperienced males, however, the males with previous experience at courting mated females spent approximately half as much time courting mated females

(ANOVA:  $F_{1,303} = 4.5$ ,  $P < 0.05$  for the interaction between male experience and female state).

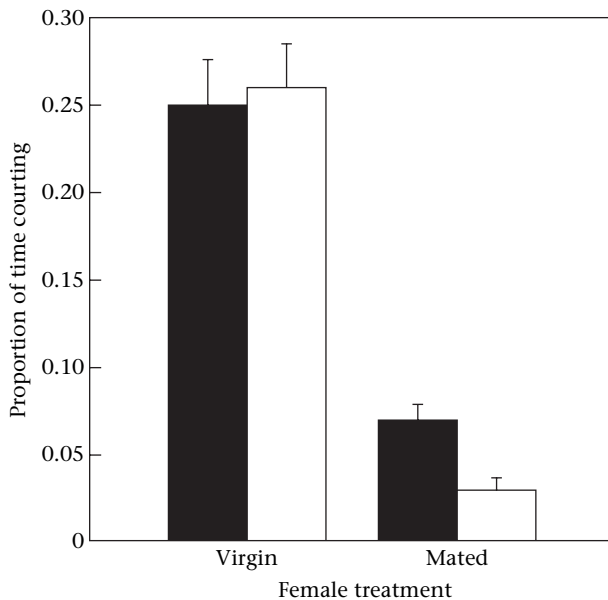
Three of the other factors included in the ANOVA, observer ( $F_{1,303} = 1.3$ ,  $P = 0.25$ ), session ( $F_{9,303} = 0.5$ ,  $P = 0.8$ ) and male treatment ( $F_{1,303} = 0.8$ ,  $P = 0.38$ ) were nonsignificant, whereas female state ( $F_{1,303} = 143$ ,  $P < 0.001$ ) and replicate effect ( $F_{3,303} = 3.9$ ,  $P < 0.01$ ) were significant. Both the frequency of matings and mating latencies were similar between male treatments. Only one inexperienced and one experienced male mated with a mated female. Seventy-one per cent of the inexperienced males and 80% of the experienced males mated with virgin females during the test period (contingency table:  $\chi^2_1 = 0.2$ ,  $P = 0.6$ ) and the mean  $\pm$  SE mating latencies were  $421 \pm 27$  s for inexperienced males and  $374 \pm 28$  s for experienced males ( $F_{1,115} = 1.3$ ,  $P = 0.25$ ).

Male experience also selectively affected the rank order of courtship latencies (Fig. 2). Compared with inexperienced males, experienced males were faster to court virgin females and slower to court mated females (Kruskal-Wallis test:  $\chi^2_3 = 27.9$ ,  $P < 0.001$ ).

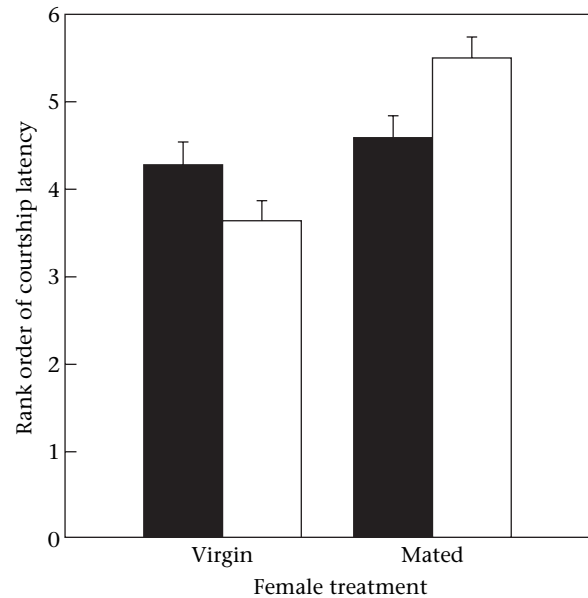
## EXPERIMENT 2: EXPERIENCE WITH MATED FEMALES VERSUS IMMATURE FEMALES

### Rationale

Males in experiment 1 may have learned to reduce courtship towards mated females because these females are unattractive. It is indeed known that even inexperienced males find recently mated females less attractive than virgin females because of differences in the pheromonal profiles of mated and virgin females (Scott 1986;



**Figure 1.** The proportion of time (mean  $\pm$  SE) that naïve males (■) and males experienced at courting mated females (□) spent courting either two virgin females or two mated females ( $N = 320$  males).



**Figure 2.** Rank order of courtship latency (mean  $\pm$  SE) for naïve males (■) and males experienced at courting mated females (□) when paired with either two virgin females or two mated females. Lower ranks correspond to shorter courtship latencies ( $N = 320$  males).

Scott et al. 1988). That is, perhaps males can only learn to further decrease the already low levels of courtship directed towards unattractive females. To further generalize the results from experiment 1, I compared courtship parameters of males experienced with courting either recently mated females or immature females. I expected the males to respond differently to their failure to mate after courting either class of female based on the difference in pheromonal composition of the two distinct female classes. Experience with soon-to-be receptive, immature females should not cause a subsequent decrease in courtship activity compared to experience with the mated females. Specifically, I predicted that (1) both male treatments would show similar high levels of courtship towards virgin females, (2) compared with males that experienced immature females, males experienced with mated females would show lower courtship towards mated females but not towards virgin females, and (3) compared with males experienced with immature females, males experienced with mated females would be slower to begin courtship of mated females but not virgin females.

## Methods

The protocol for experiment 2 was similar to that of experiment 1 and is presented here briefly while focusing on novel features. After I sexed the flies, I placed the males individually in vials and kept the females 30 per vial. The experiment had four replicates conducted on different days, each consisting of six sessions, with each session including eight males. Hence, I tested a total of 192 males. The experimental protocol consisted of all four combinations of two male treatments during the experience phase (experience with mated females or experience with immature females), and two female states presented during the subsequent test phase (mated or virgin females).

### Experience phase

Each session, I randomly selected four males and placed them individually into vials, each containing two mated females. The other four randomly selected males were placed individually in vials each containing two immature females. Ninety-nine per cent of the males courted the females during the experience phase, with courtship typically commencing within 2 min. Time constraints did not allow me to measure exact courtship duration during the experience phase. However, the results reported in experiment 1 for inexperienced males (Fig. 1) provide a good approximation of the proportions of time that males spent courting. On average, male fruit flies spend longer proportions of time courting immature and mature virgin females than they do mated females (Manning 1967; Tompkins et al. 1983). The experience phase lasted 60 min.

### Test phase

At the end of the experience phase, I transferred each of the eight males into a fresh, empty vial. Then (1) two males experienced with mated females each received two virgin females, (2) two males experienced with mated

females each received two mated females, (3) two males experienced with immature females each received two virgin females, and (4) two males experienced with immature females each received two mated females.

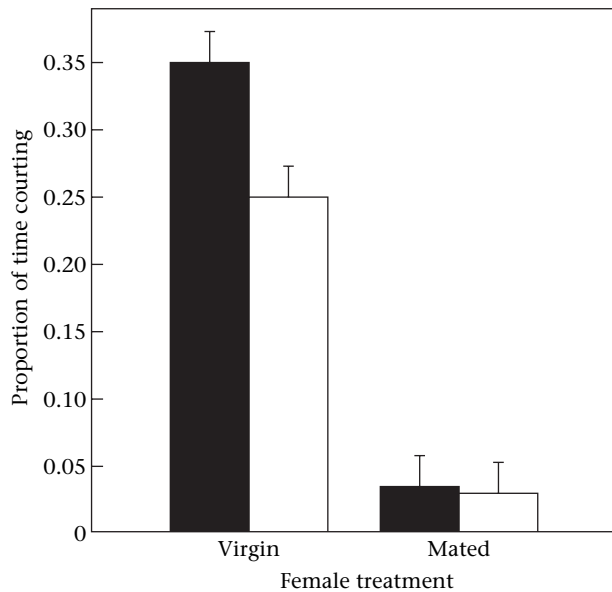
Two trained observers that were blind to male and female treatments each conducted 15-min observations of four randomly selected vials containing (1) two virgin females and a male experienced with mated females, (2) two virgin females and a male experienced with immature females, (3) two mated females and a male experienced with mated females, (4) two mated females and a male experienced with immature females. Each observer recorded the start and end of each male's courtship activity, and the start of mating if it occurred.

## Analyses

The main statistical analysis was done on arcsine-transformed proportions of the time spent courting out of the total time available. Three test trials in which mating occurred with supposedly previously mated females were excluded from the analysis because I assumed that these females had not completed successful mating on the previous day. In trials with no matings (100% of the trials with mated females and 34% of the trials with virgin females), the total time available for courtship was 15 min. In trials with matings (66% of the trials with virgin females), the time available for courtship was the time prior to mating. I also examined the courtship latency, defined as the duration from the start of each session until the first courtship activity by each male. Males that did not court were assigned a courtship latency of 15 min, which was the overall duration of the test phase. A single additional male was removed from this analysis because of a computer timing error. The statistical tests did not violate ANOVA assumptions.

## Results

Male experience significantly affected both the proportion of time males spent courting and courtship latency. All my three predictions, however, were falsified. First, compared with males experienced with mated females, the males experienced with immature females spent more time courting virgin females. Second, males in both treatments showed similar, low courtship of mated females (Fig. 3). An ANOVA on the proportion of time spent courting revealed a significant effect of both male experience ( $F_{1,176} = 5.6$ ,  $P < 0.02$ ) and test female ( $F_{1,176} = 181.3$ ,  $P < 0.001$ ) but the interaction between the two factors was nonsignificant ( $F_{1,176} = 2.1$ ,  $P = 0.15$ ). An ANOVA including only the mated test females revealed no significant difference between male treatments ( $F_{1,88} = 1.1$ ,  $P = 0.28$ ), whereas an ANOVA on the virgin test females showed a significant effect of male experience ( $F_{1,91} = 4.5$ ,  $P < 0.05$ ; Fig. 3). The ANOVA including the whole data set also revealed a significant replicate effect ( $F_{3,176} = 3.8$ ,  $P < 0.02$ ) and nonsignificant effects of observer ( $F_{1,176} = 0.17$ ,  $P = 0.68$ ) and session ( $F_{5,176} = 1$ ,  $P = 0.4$ ).



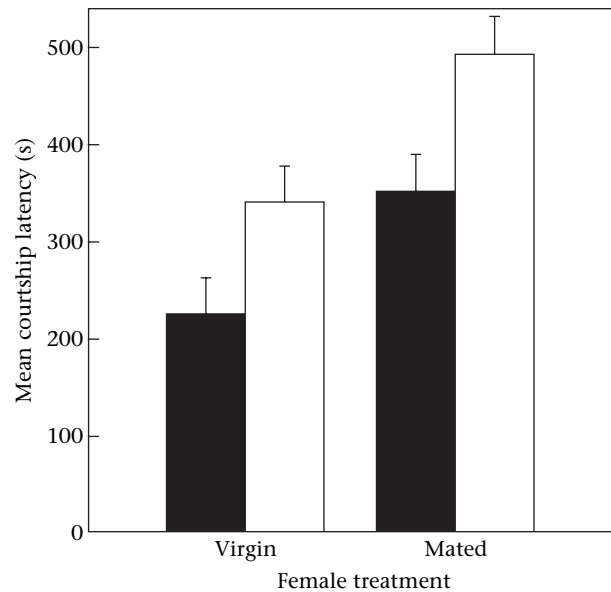
**Figure 3.** The proportion of time (mean  $\pm$  SE) that males experienced at courting immature females (■) and mated females (□) spent courting either two virgin females or two mated females ( $N = 192$  males).

Of the males experienced with mated females, 94% courted virgin females and 71% courted mated females. Among the males experienced with immature females, 96% courted virgin females and 88% courted mated females. Both the frequency of mating and mating latency were similar between the male treatments. Sixty-five per cent of the males experienced with mated females and 69% of the males experienced with immature females mated with virgin females during the test period ( $\chi^2_1 = 0.19$ ,  $P = 0.8$ ), with mean  $\pm$  SE mating latencies of  $499 \pm 32$  s for males experienced with mated females and  $464 \pm 31$  s for males experienced with immature females ( $F_{1,54} = 0.36$ ,  $P = 0.5$ ).

Finally, compared with the males experienced with immature females, the males experienced with mated females were slower to begin courtship of both mated females and virgin females ( $F_{1,175} = 11.5$ ,  $P < 0.001$ ; Fig. 4). Males in both treatments were faster to begin courting virgin than mated females ( $F_{1,175} = 13.6$ ,  $P < 0.001$ ), but the interaction between male treatment and female category was nonsignificant ( $F_{1,175} = 0.1$ ,  $P = 0.7$ ).

## DISCUSSION

In both experiments, experience affected the courtship behaviour of male fruit flies. In experiment 1, compared with inexperienced males, the males experienced at courting mated females took longer to initiate courtship towards newly encountered mated females and courted these females for shorter durations. Although experienced males initiated courtship towards virgin females sooner than did inexperienced males, males in both treatments spent similar times courting virgin females (Figs 1, 2).



**Figure 4.** The mean courtship latency for males experienced at courting immature females (■) and mated females (□) when paired with either two virgin females or two mated females ( $N = 192$  males). A lower mean implies that the males were quicker to court.

These results cannot be explained by some general change in the motivational state of experienced males. Rather, the results suggest a specific change in behaviour towards mated females based on learning. The involvement of learning in courtship behaviour has been well documented in the neurogenetic literature. Wild-type fruit flies show learning in the context of courtship, but neither learning mutants nor flies with mushroom body ablations show such learning (Siegel & Hall 1979; Tompkins et al. 1983; McBride et al. 1999).

In experiment 2, compared to males experienced with mated females, males experienced with immature females decreased courtship latency towards virgin and mated females and increased courtship duration towards virgin females. Why? It appears that inexperienced males are willing to court females with a wide variety of pheromonal composition. This is probably an adaptive behaviour because pheromonal composition varies greatly in time and place (Jallon & David 1987). That is, inexperienced males may not be able to accurately classify females based on their pheromones. Experience with immature females, however, probably provides the males with some baseline information about the pheromonal compositions of the local females. Such experience might cause males to subsequently find mated females unattractive, because mated females release much higher levels of the antiaphrodisiac, 7-tricosen, compared with immature females (Scott 1986; Scott et al. 1988). In contrast, the pheromonal composition of mature virgin females is virtually identical to that of immature females. Because immature females become sexually receptive within several hours, males experienced with immature females may show increased tendencies to approach any female and to court virgin



females longer than would males experienced with mated females. Results of experiment 1 are also in agreement with the general proposition that male experience with pheromonal compositions of females allows them to refine their subsequent courtship behaviour. This scenario may be critically tested through experimental manipulation of pheromones (Antony et al. 1985; Scott et al. 1988).

Results of both experiments indicated that fatigue, habituation or motivational changes could not explain male behaviour. In experiment 1, males experienced with mated females selectively reduced courtship towards mated but not towards virgin females (Fig. 1). In experiment 2, even though males in both treatments courted but did not mate, the males' experiences had distinct effects depending on the type of female they courted (Figs 3, 4). Finally, in experiment 2, males experienced with immature females showed low levels of courtship towards mated females (Fig. 3). This indicates that the males' experience with immature females did not cause an indiscriminate increase in courtship behaviour.

Male fruit flies typically face the challenging task of attempting to obtain mates from among many individuals of several species and a variety of ages and levels of sexual receptivity. Moreover, most intraspecific females may be unreceptive because they have already mated in recent days (Spieth & Ringo 1983). Finally, individual females show large genetically and environmentally based variation, which may obscure differences indicating receptivity (Jallon & David 1987). To some extent, the males rely on innate responses to female characteristics, especially pheromonal composition. For example, even naïve male *D. melanogaster* spend more time courting virgin females than they do mated females (Fig. 1). The males also spend more time courting younger virgin females than they do older virgin females, and they spend more time courting females that are several days postmating than they do females that are one day postmating (Cook & Cook 1975). The results presented here, however, indicate that, in addition to responding to relatively clear categories of receptive and unreceptive females, the males can refine their courtship behaviour based on experience. Experience may result in males increasing the proportion of time they spend courting receptive females, and heightening searching and courting after encountering immature females, who mature within a short time. Among insects, learning in the context of courtship has also been documented in a few species of solitary bees, in which the males learn to avoid unreceptive individual females based on individually specific odours (Barrows 1975; Smith 1983; Wcislo 1992). Learning in the context of courtship is better known in vertebrates (e.g. Schwagmeyer 1995; Hollis et al. 1997; Mills et al. 1997; Freeberg et al. 2002).

How might learning in the context of courtship affect male fitness? In a laboratory study with the *Dunce* mutant, which diminishes fly learning, Gailey et al. (1985) documented a significant mating advantage of wild-type flies over *Dunce* flies when placed together with one virgin female and nine immature males. Only the wild-type flies could learn to avoid courtship of the immature males, allowing them to selectively court the virgin female. Gailey et al. (1985), however, found no difference in

mating frequencies of wild-type flies and *Dunce* flies either when the two fly types were placed together with only a single virgin female, or when each male was placed singly with a single female. Thus, because males in the present experiment were tested individually, the data for the proportion of time courting and the frequency of mating cannot inform us about the fitness effect of learning in males under natural settings in which many males search simultaneously for rare receptive females among numerous unreceptive flies. For example, a study in which male *D. melanogaster* reared in an enriched environment showed a 2:1 mating advantage over conspecific males reared in a standard environment was well replicated only when males of each type competed for a virgin female. When each male was placed singly with a virgin female, however, mating frequencies of the two male types were virtually identical (Dukas & Mooers 2003). Furthermore, because male courtship is negatively associated with longevity (Cordts & Partridge 1996), selective courtship could be associated with higher male fitness.

My experiments do not critically control for the possibility that the test females sensed differences in male experience and behaved differently towards experienced and inexperienced males, which, in turn, influenced males' courtship. Although it is unlikely that females control male courtship, mature females are known to have almost complete control of mating (Spieth & Ringo 1983; Markow 2000). The results of the present experiments, however, indicated no difference in mating frequency between the two male treatments, showing that females did not discriminate between male treatments. Given that no discrimination by females occurred in the mating stage, it is unlikely that such discrimination affected courtship. For comparison, in a previous set of experiments with the same fly population, males reared under environmental enrichment or standard conditions showed similar courtship latencies and courtship durations, but males from the enriched environment showed a 2:1 mating advantage (Dukas & Mooers 2003). Similarly, Markow et al. (1978) documented similar courtship latencies and courtship durations, but a significant mating advantage for virgin males over recently mated males.

The adaptive value of an experience-based change in courtship behaviour in male fruit flies is not obvious: although males can benefit from avoiding or reducing their courtship effort towards classes of unreceptive females, males must also ensure that they do not mistakenly learn to avoid a whole class of receptive females after a chance encounter with a receptive female that rejects their courtship effort, which is a rather common occurrence. Hence, the adaptive value of learning in the domain of courtship must be evaluated by considering all relevant parameters and potential benefits and costs, including the frequency of receptive conspecific females, the difference in pheromones and behaviour between receptive and unreceptive females, and the benefit of reducing the proportion of time spent courting less receptive females. I am currently examining these issues.

## Acknowledgments

I thank S. Balshine, J. Galef, D. Papaj and two anonymous referees for comments on the manuscript, S. Cope and P. Henry for assistance, and the Natural Sciences and Engineering Research Council of Canada and the Faculty of Science, McMaster University for financial support.

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