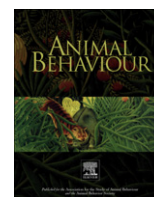




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## Causes and consequences of male–male courtship in fruit flies

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The mechanisms maintaining homosexual behaviour in animals are not well understood. In fruit flies, where male–male courtship is prevalent, it has been suggested that young males gain from being courted by mature males, perhaps through learning. I conducted two series of experiments to critically examine why mature males court immature males and what immature males may gain from such courtship. The results indicate that mature males do not identify the sex of the sexually ambiguous immature males and find them attractive even after substantial experience. These findings agree with recent research indicating that males initially court a broad range of potential mating targets and then narrow their courtship focus based on their experience of being rejected by classes of flies such as recently mated females or heterospecific females, which are clearly identified by their distinct pheromonal profiles. Compared to inexperienced males, males that either had received courtship when immature or had been housed with mature males when young did not have either higher mating frequencies or shorter mating latencies but spent more time courting females. Such higher courtship intensities may translate into a mating advantage in some settings. It appears that young males that interact with mature males develop into a sexually aggressive phenotype, which could better prepare them to compete for females.

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Homosexual behaviour is widespread among animals including humans, but the proximate and ultimate mechanisms that have maintained it in spite of its probable costs are not well understood (Gavrillets & Rice 2006; Bailey & Zuk 2009). In fruit flies, *Drosophila melanogaster*, mature males intensely court immature males (Jallon & Hotta 1979; McRobert & Tompkins 1983). Intriguingly, McRobert & Tompkins (1988) suggested that immature male fruit flies that elicit courtship from mature males subsequently gain increased mating success owing to mechanisms 'reminiscent of the song learning process that occurs in some bird species' (McRobert & Tompkins 1988, page 1096). This tantalizing claim, however, has not been subjected to the necessary critical research.

Learning in the context of sexual behaviour is well known in fruit flies as well as other insects (Siegel & Hall 1979; Dukas 2006). In fruit flies, males initially court a broad range of receptive and unreceptive conspecific and heterospecific females. The males then learn to selectively reduce courtship of the female categories that persistently reject them, thus allowing them to maximize the time they devote to courting sexually receptive females (Dukas 2004b, 2005, 2009; Dukas et al. 2006). The phenomenon of males

courting immature males readily fits within the above generalization: immature males do not yet produce the male pheromone, cis-vaccenyl acetate (cVA), and generate other compounds that attract males (Curcillo & Tompkins 1987; Vaia et al. 1993). Hence the mature males, which initially adopt a broad courting strategy, also court the sexually ambiguous immature males. Mature males, however, do not court young males older than approximately 24 h, which produce the male pheromone cVA (Curcillo & Tompkins 1987). Furthermore, mature males experienced with immature males spend less time courting other immature males than do inexperienced mature males (Gailey et al. 1982; McRobert & Tompkins 1988).

While we can readily understand why mature males initially court immature males, the suggestion that the immature males gain and perhaps even learn from such courtship is puzzling. McRobert & Tompkins (1988) only reported a significant reduction in mating latency from about 12 to 6 min in males that had received courtship versus those that had not received courtship when immature. As far as I know, no further data about this potentially important phenomenon have been published. Thus it is still unclear how the experience of being courted alters a young male's behaviour and what immature males may learn from mature males. To resolve these intriguing issues, I conducted two series of experiments examining the causes and consequences of male–male courtship in fruit flies. Specifically, I asked (1) whether mature males would initially court but rapidly learn to avoid immature

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males, (2) whether males that receive courtship by mature males would later have higher mating rates and shorter mating latencies than age-matched males that do not receive mature-male courtship, and (3) what mechanisms could mediate the possible mating advantage of males that receive courtship when immature.

## GENERAL METHODS

I used Canton-S *D. melanogaster* kept in  $20 \times 20 \times 35$  cm population cages containing a few thousand individuals inside an environmental chamber at 25 °C and 60% relative humidity, on a 12:12 h light:dark cycle. These flies are different from the wild-caught *D. melanogaster* I used in previous work (e.g. Dukas 2005). Each population cage had two standard 240 ml food bottles each containing 50 ml of standard fly medium made of water, corn meal, glucose, yeast, sucrose, agar and methyl paraben. The flies used in the experiment were developed at a low density in food bottles each containing about 200 larvae. I collected sexually immature flies and placed them in single-sex food vials as detailed below for each experiment. All flies were used only once in the experiments. The seven experiments below are sorted into two categories, with series 1 focusing on the effect of experience on the mature males and series 2 dealing with the effect of experience on the immature males.

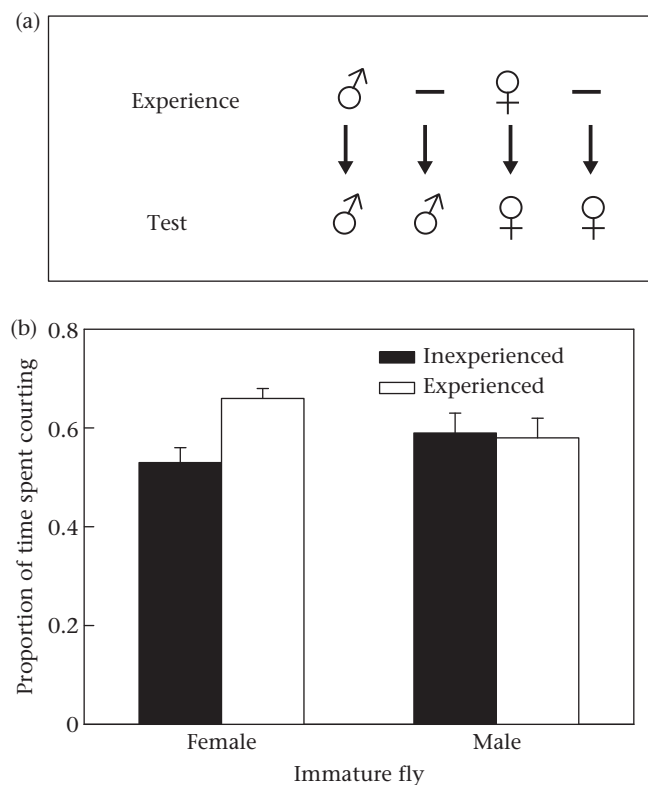
### EXPERIMENT 1A: DO MATURE MALES INITIALLY COURT AND THEN LEARN TO AVOID COURTING IMMATURE MALES?

First, I wished to verify the prevalence and magnitude of male–male courtship and test whether mature males are quick to reduce courtship of immature males. To distinguish between possible male fatigue and learning, I also compared males' willingness to court immature females over time. I predicted that the males would show rapid decline in courting immature males but not immature females, which are highly sexually attractive (e.g. Dukas 2005).

#### Methods

On day 1, I sexed focal males within 8 h of eclosion and placed them 20 per food vial. On day 4, I moved the focal males individually into vials because male isolation increases their responsiveness to potential mates (Noor 1997; R. Dukas, unpublished data). On day 5, I sexed immature males within 2 h of eclosion and marked them by clipping the tips of their wings. Wing clipping is a standard marking technique in fruit flies, which has no effect on fly behaviour (e.g. Partridge & Farquhar 1983). I also sexed immature females within 17 h of eclosion.

The experiment consisted of a 30 min training phase after which I transferred the males into fresh vials for a 15 min break. Then I moved the males into new vials for a 15 min test. I had four groups of mature males (Fig. 1a). Group 1 males were each placed in a vial containing two immature males and later tested with two new immature males. Group 2 males were each placed in an empty vial and later tested with two new immature males. Group 3 males were each placed in a vial containing two immature females and later tested with two new immature females. Group 4 males were each placed in an empty vial and later tested with two new immature females. Observers blind to fly treatment recorded all courtship activity in the test vials on laptops equipped with a custom-made program. Courtship behaviour included following, wing vibrations and mounting attempts. Later, I calculated for each male the proportion of time spent courting during the test (e.g. Dukas 2005) and conducted statistical analyses on the arcsine square-root values, which met ANOVA's assumptions. I tested



**Figure 1.** (a) Protocol used in experiment 1a. Mature males either experienced immature males or had no experience and were then tested with new immature males. Other mature males either experienced immature females or had no experience and then were tested with new immature females. (b) Average  $\pm$  SE proportion of time males spent courting either immature females or immature males in the test following the protocol in (a).  $N = 188$  males.

a total of 188 males. All mature males in groups 1 and 3 courted during training and all but three (two of the immature male treatment and one of the immature female treatment) courted during the test.

#### Results

Regardless of training, the males spent almost as much time courting immature males as they did immature females (Fig. 1b). Contrary to my prediction, however, males that had courted immature males for 30 min subsequently spent similar times courting immature males as did inexperienced males ( $F_{1,93} = 0.03$ ,  $P = 0.9$ ). On the other hand, as expected, males that had courted immature females for 30 min subsequently spent significantly longer times courting immature females than did inexperienced males ( $F_{1,91} = 13.6$ ,  $P < 0.001$ ; Fig. 1b).

### EXPERIMENT 1B: DO MALES LEARN TO AVOID COURTING IMMATURE MALES WITH PROLONGED EXPERIENCE?

Experiment 1a indicated intense courtship of immature males and no reduction in such courtship after 30 min of experience compared to control males. I thus conducted another experiment in which males had 60 min of courtship experience. This experiment involved a repeated measures protocol in which I compared courtship by the same males before and after gaining experience. The repeated measures protocol was more powerful than the between-male protocol in detecting changes with experience in a recent set of experiments (Dukas 2009).

## Methods

The experiment was similar to experiment 1a with the following exceptions. All the males received 1 h training. I placed half the males in vials each containing two immature males marked with white fabric paint (Duncan Enterprises, CA, U.S.A.) on their thoraxes and the other half of the males in vials each containing two immature females. I switched the marking technique from wing clipping to paint, which I had previously used with no ill effects (Dukas & Mooers 2003), to verify that the wing clipping in experiment 1a was not responsible for the unexpected results.

At the end of the 1 h training, I transferred the males into empty vials for a 15 min break, after which I placed each male trained with immature males with two new immature males and each male trained with immature females with two new immature females. Observers blind to fly treatment recorded all courtship activity during the first 15 min of the training phase and during the test. I tested 48 males, half trained and tested with immature males and the other half trained and tested with immature females. All males courted during training and all but two (one of each treatment) courted during the test.

## Results

Males courting immature males showed a similar decline in courtship with experience to that of males courting immature females (repeated measures ANOVA: treatment  $\times$  trial interaction:  $F_{1,46} = 0.03$ ,  $P = 0.9$ ; Fig. 2a). That is, there was no evidence for the predicted decline in courting immature males relative to the control. The males trained and tested with immature females spent more time courting than the males trained and tested with immature males ( $F_{1,46} = 14.3$ ,  $P < 0.001$ ), and both treatments showed a decline in the proportion of time spent courting after the 1-h experience (within-subject effect:  $F_{1,46} = 12.3$ ,  $P < 0.001$ ; Fig. 2a).

### EXPERIMENT 1C: COMPARING THE EFFECT OF MALES' EXPERIENCE WITH IMMATURE MALES AND RECENTLY MATED FEMALES

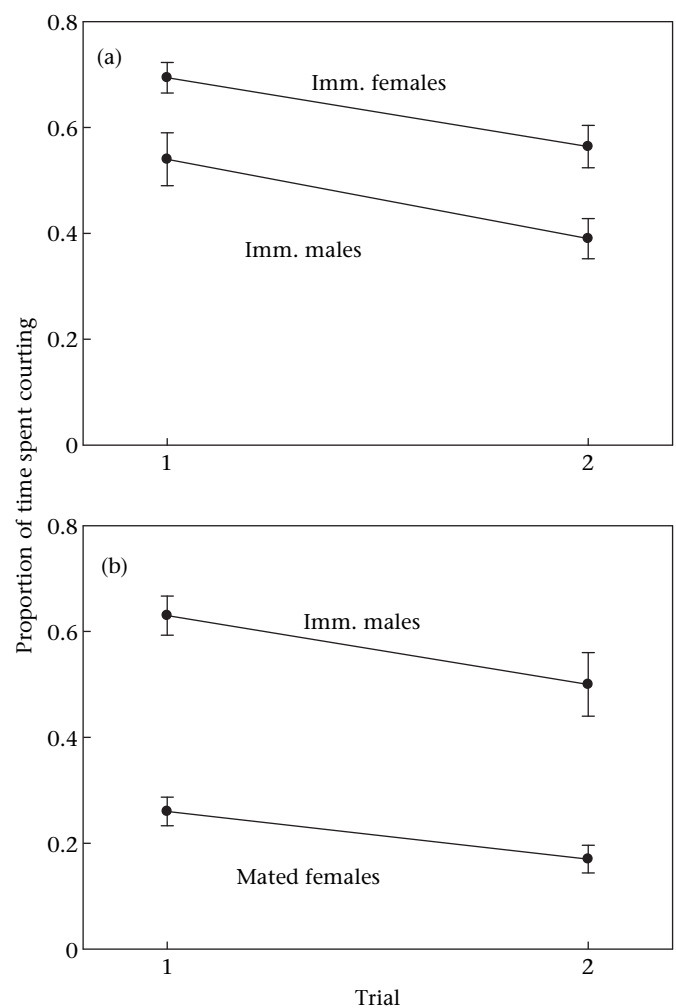
Experiment 1b indicated intense courtship of immature males and only moderate reduction in such courtship after 60 min of experience. To place these results in a broader perspective, I conducted another experiment in which I compared the effects of experience on courtship of immature males versus recently mated females. Based on previous data from wild-caught *D. melanogaster*, I expected that, with experience, the males would decrease courtship of the recently mated females (Dukas 2005). I wished to compare that reduction to the expected decrease in courting immature males with experience.

## Methods

The experiment was very similar to experiment 1b with the exception that I used recently mated females rather than immature females. The 5-day-old females were placed each with two males 1 day before the test and observed to complete mating. I tested 45 males, 24 trained and tested with immature males and 21 trained and tested with recently mated females.

## Results

Males courting immature males showed a decline in courtship with experience similar to that of males courting recently mated females (repeated measures ANOVA: treatment  $\times$  trial interaction:  $F_{1,43} = 0.04$ ,  $P = 0.8$ ; Fig. 2b). That is, again, there was no evidence



**Figure 2.** (a) Average  $\pm$  SE proportion of time males spent courting either immature females or immature males during the first 15 min period of an hour-long training (trial 1), and during a subsequent 15 min test period (trial 2).  $N = 48$  males. (b) Average  $\pm$  SE proportion of time males spent courting either recently mated females or immature males during the first 15 min period of an hour-long training (trial 1), and during a subsequent 15 min test period (trial 2).  $N = 45$  males.

for the predicted decline in courting immature males relative to the control. The males trained and tested with immature males spent more time courting than the males trained and tested with recently mated females ( $F_{1,43} = 52$ ,  $P < 0.001$ ), and both treatments showed a decline in the proportion of time spent courting after the 1-h experience (within-subject effect:  $F_{1,43} = 10.8$ ,  $P < 0.005$ ; Fig. 2b).

### EXPERIMENT 2A: DO MALES GAIN FROM BEING COURTED WHEN IMMATURE?

Here I wished to establish whether immature males that receive courtship indeed benefit from that experience. I thus compared the mating success and mating latency of males that either had or had not received courtship while immature. In order to understand the possible differences between the male treatments in mating parameters, I also quantified their courtship behaviour.

## Methods

### Training of model males

In this experiment, I first prepared model males and then allowed them to court the focal males. On day 1, I sexed model

males within 2 h of eclosion, marked them with a dot of white paint on their thoraxes and placed them singly in food vials each containing three mature males collected from the regular population cages. All the immature model males received courtship during their first hour in the vials. On the morning of day 4, I removed the mature males from the model males' vials and added to each vial two virgin 4-day-old females. Preliminary data and information presented below indicated close to 100% mating rates by the mature females. I removed the females on the afternoon of day 4. In short, each model male received courtship when immature and courted and mated with females when mature.

#### Training of focal males

On day 5, I sexed focal males within 2 h of eclosion and placed them individually in food vials. Half the vials belonged to the inexperienced treatment and contained no other flies. The other half of the vials constituted the experienced treatment and each contained two model males. During their first hour in the vials, all focal males of the experienced treatment received at least one bout of courtship and most received over five courtship bouts. I removed the model males from vials of the experienced treatment on day 8.

#### Test

On the morning of day 9, I moved each focal male into a vial containing two virgin 4-day-old females. Observers blind to fly treatment recorded all courtship behaviour for 15 min and all matings occurring within 30 min. I then calculated the proportion of time spent courting out of the total time available prior to mating, if mating occurred (e.g. [Dukas 2005](#)). I tested a total of 165 males.

#### Results

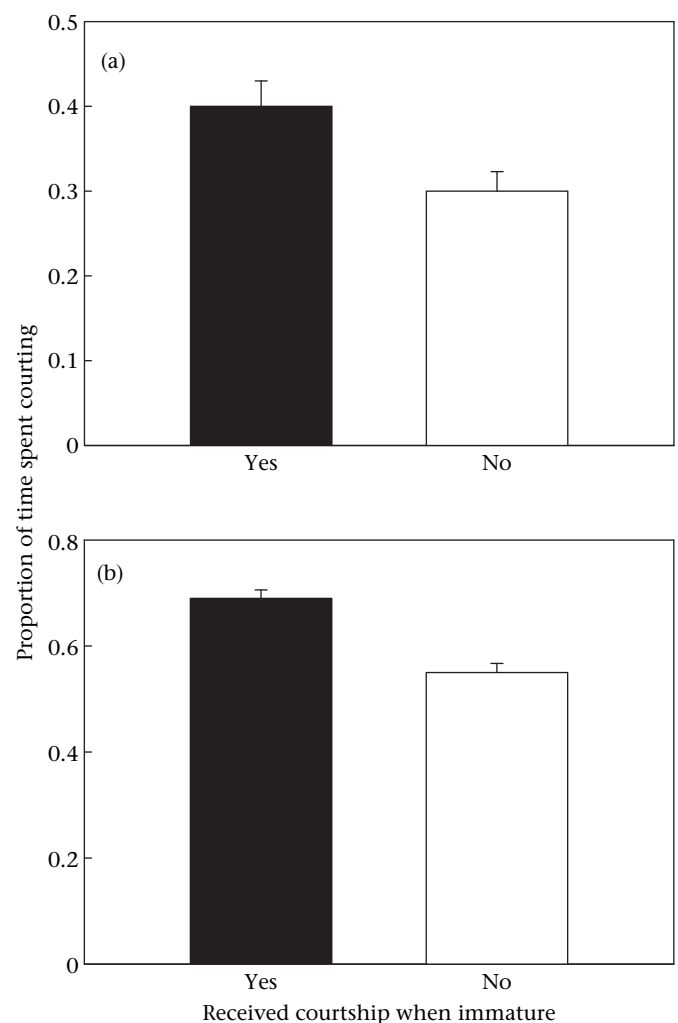
All focal males except for four males from the experienced treatment mated during the test. The mating latencies of experienced and inexperienced focal males were similar (mean  $\pm$  SE:  $355 \pm 47$  and  $292 \pm 30$  s, respectively;  $F_{1,159} = 0.01$ ,  $P = 0.9$ ). The courtship latencies of the two male treatments were also similar ( $125 \pm 21$  and  $128 \pm 17$  s, respectively;  $F_{1,159} = 1.9$ ,  $P = 0.17$ ). On the other hand, the experienced males spent a greater proportion of time courting females than did the inexperienced males ( $F_{1,159} = 5.3$ ,  $P < 0.05$ ; [Fig. 3a](#)).

#### EXPERIMENT 2B: DO MALES GAIN FROM BEING COURTED WHEN IMMATURE? ANOTHER TEST USING YOUNGER, MORE SELECTIVE FEMALES

In experiment 2a, the males that received courtship when immature courted females significantly more than the males that did not receive courtship when immature. The females, however, were highly receptive, as indicated by the 98% mating rate and the brief average mating latency of approximately 5 min. To critically test whether the clear behavioural difference between the male treatments would translate into a mating advantage, I repeated experiment 2a using young, sexually mature females, which are more selective.

#### Methods

The protocol was similar to that of experiment 2a with the exception that I used females that were 21–25 h old. I tested a total of 172 males.



**Figure 3.** Average + SE proportion of time males that either received or did not receive courtship while immature spent courting (a) 4-day-old virgin females ( $N = 165$  males) and (b) 1-day-old virgin females ( $N = 172$  males).

#### Results

The frequencies of matings were similar among the experienced and inexperienced focal males (55% and 49%, respectively; logistic regression: Wald test:  $\chi^2_1 = 0.6$ ,  $P = 0.5$ ). The mating latencies of the experienced and inexperienced focal males were also similar ( $588 \pm 66$  and  $614 \pm 72$  s, respectively;  $F_{1,83} = 0.1$ ,  $P = 0.7$ ). However, as observed in experiment 2a, the experienced males spent a greater proportion of time courting females than did the inexperienced males ( $F_{1,164} = 32$ ,  $P < 0.001$ ; [Fig. 3b](#)). This pattern was similar among the males that mated and the ones that failed to mate (treatment  $\times$  mated interaction:  $F_{1,164} = 2.4$ ,  $P = 0.12$ ).

#### EXPERIMENT 2C: MALE–MALE COMPETITION

Experiments 2a and 2b indicated that experienced males were more persistent in their courtship than inexperienced males but that this difference did not translate into a mating advantage using a no-choice protocol in which each test vial contained a single male. I thus switched to a male–male competition protocol in which two males, one of each treatment, competed for a single female. Such a protocol could help reveal a possible mating advantage of the



experienced males, which may result either from female choice or male–male interactions.

### Methods

The protocol was similar to that of experiment 2b with a few modifications. The mature model males were marked with a pink fluorescent powder 1 day prior to using them. By the time they courted the immature focal males, the mature model males groomed the powder from much of their bodies. This marking technique has no ill effects on either fly behaviour or survival (Crumpacker 1974). Upon eclosion, half the focal males of each treatment were marked on their thoraxes with a dot of white fabric paint. For the test, I used females that were 24–34 h old, which are young but sexually mature. I added to each female vial one male of each experience treatment, with half the vials having marked experienced males and the other half containing marked inexperienced males. Observers blind to male treatment recorded the identities of the mated males. I tested a total of 350 male pairs and recorded 332 matings (95%) within 2 h.

### Results

There was no difference in the mating frequencies of the experienced and inexperienced males (54% versus 46%, respectively; log linear analysis:  $\chi^2_1 = 1.7$ ,  $P = 0.19$ ), but unmarked males had a significant mating advantage over marked males (60% versus 40%; log linear analysis:  $\chi^2_1 = 14.9$ ,  $P < 0.001$ ). The mating latencies of the experienced and inexperienced focal males were similar ( $1286 \pm 102$  and  $1392 \pm 109$  s, respectively;  $F_{1,328} = 0.35$ ,  $P = 0.55$ ), and marking had no significant effect on mating latencies ( $F_{1,328} = 1.4$ ,  $P = 0.24$ ).

### EXPERIMENT 2D: EFFECTS OF MATURE-MALE COURTSHIP VERSUS PRESENCE

In experiments 2a and 2b, I found that males that had received courtship from mature males when immature spent more time courting females than males that had not experienced such courtship. However, it was not clear whether the key factor was active courtship by mature males or merely the presence of mature males. To resolve this issue, I compared courtship by (1) focal males that had received courtship when immature, (2) focal males that had only spent time with mature males when mature and (3) focal males with no experience with mature males. Because I had found no mating advantage as a function of experience in experiments 2a and 2b, I used sexually immature females. This allowed me to eliminate variation caused by female receptivity and to control for the possibility that variation in feedback from the females affected male courtship.

### Methods

The general methods were similar to those in experiments 2a and 2b except that I had three treatments. On day 1, focal males were placed individually in vials upon eclosion. Focal males of the courtship-experience treatment each received three mature males on day 1 and all were observed to be courted. I removed the mature males on day 3. Focal males of the mature-male-presence treatment each received three mature males on day 2. The focal males were not courted by the mature males but they occasionally approached and were approached by the mature males. Two-day-old males possess the adult male pheromone and other males recognize them as adult males (Curcillo & Tompkins 1987).

I removed the mature males on day 4. The third treatment involved focal males housed alone until the test.

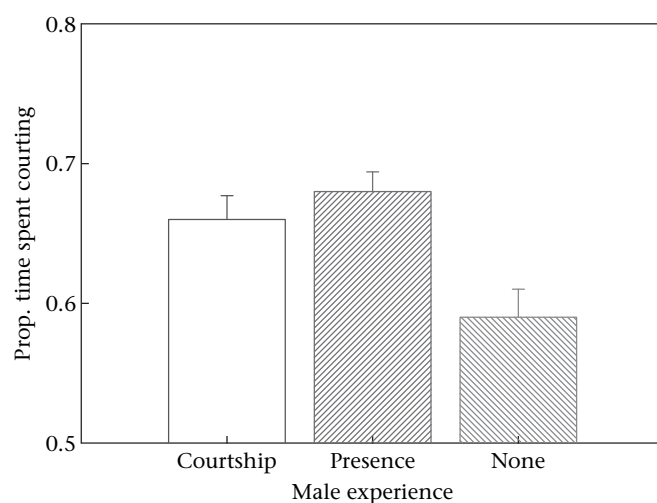
On the morning of day 5, each focal male received two immature females younger than 20 h, and observers blind to fly treatment recorded the males' courtship behaviour. I tested a total of 192 males and conducted two a priori contrasts, one comparing the focal males experienced with mature males to the inexperienced focal males and the other comparing the focal males experienced with courtship by mature males versus presence of mature males.

### Results

The males' experience strongly affected their courtship intensity ( $F_{2,189} = 7.9$ ,  $P < 0.001$ ; Fig. 4). The males that had spent 2 days with mature males courted more than the males that had been alone ( $t_{189} = 3.8$ ,  $P < 0.001$ ), but there was no difference in courtship duration between the males that had been courted versus the males that had merely shared vials with mature males ( $t_{189} = -1.0$ ,  $P = 0.3$ ).

### DISCUSSION

The results of my first series of experiments confirmed earlier reports indicating that male *D. melanogaster* find newly eclosed males highly sexually attractive (Jallon & Hotta 1979; McRobert & Tompkins 1983). The males courted the immature males almost as much as they courted sexually immature females (Figs 1, 2a) and much more than they courted recently mated females (Fig. 2b), which, though typically unreceptive, do remate at a low frequency if pursued persistently (about 10% in 2 h trials with females 1–3 days after mating in my laboratory population; R. Dukas, unpublished data). Contrary to previous reports using either anaesthetized (Gailey et al. 1982) or active immature males (McRobert & Tompkins 1988), however, I found that the mature males did not reduce courtship towards immature males after 30 min of experience compared to control, inexperienced mature males (Fig. 1b). This discrepancy may reflect differences in fly strains and protocols. Most notably, I always used immature males 1–2 h posteclosion while McRobert & Tompkins (1988) used immature males up to 9 h old. Data in Curcillo & Tompkins (1987) indicate that immature males gradually receive less courtship when they are over 4 h old,



**Figure 4.** Average  $\pm$  SE proportion of time males that (1) received courtship while immature, (2) shared a vial with mature males while mature, and (3) encountered no other males spent courting sexually immature females.  $N = 192$  males.

suggesting that mature males may be more likely to identify the sex of older immature males with experience.

As noted in the Introduction, the prevalence of male–male courtship in fruit flies should not be surprising. Given the large spatial and temporal variation in the pheromonal profiles of fruit flies (Jallon & David 1987; Ferveur et al. 1996), it seems appropriate for inexperienced males to initially court all flies that do not obviously belong to unreceptive categories. Theoretical analyses indeed indicate that the male fruit flies' strategy of initially courting broadly and then learning to narrow their courtship targets is optimal over alternatives under a realistic range of parameter values (Dukas et al. 2006). It is perhaps surprising, however, that the males failed to identify the sex of immature males even after persistent physical contact and frequent mountings in spite of the dramatic sex differences in genital morphology, which are evident even in newly eclosed flies. Another surprising result is that the males found sexually immature males much more attractive than recently mated females (Fig. 2b) even though the males could gain fitness only from courting the latter category. Recent analyses indeed indicate that the ambiguous state of flies with no pheromones is more attractive to male fruit flies than the pheromonal profile of a virgin female (Billeter et al. 2009). This provides a proximate, though not ultimate, explanation for my results.

A final issue regarding the mature-males' behaviour involves the cost they incur from courting immature males. Although earlier studies implied that the cost may be low owing to rapid habituation to the immature males (McRobert & Tompkins 1988), in both McRobert & Tompkins (1988) and my study (Figs 1, 2), the mature males experienced with immature males still spent 50% or more of their time courting immature males. Because the recruitment rate of virgin females and immature males is similar, and most sexually mature females are typically mated (e.g. Markow 1988), the proportion of time mature males could spend pursuing immature males may not be negligible. Field data are necessary to clarify this point.

My second series of experiments clearly indicated that males that had received courtship when immature did not mate at a higher frequency, nor did they mate faster than males that had not received courtship when immature. In contrast, in three experiments, I found that males that had received courtship when immature spent a higher proportion of their time courting females than males that had not received courtship when immature (Figs 3, 4). Thus, there was a robust behavioural difference in the males' sexual behaviour as a function of experience. It is possible that, under some conditions, higher courtship intensities and persistence would translate into a mating advantage.

Courtship by mature males of immature males, however, was not necessary for generating the behavioural difference between males, as indicated by the fact that young males that had been housed with mature males showed a higher courtship intensity similar to that exhibited by the males that had received courtship after eclosion (Fig. 4). That is, my data do not corroborate earlier speculations that immature males learn specific courtship parameters from mature males (McRobert & Tompkins 1988). The simplest interpretation of my results is that young males that experience mature males develop into a more competitive mature-male phenotype than young males that develop alone. Similar phenotypic plasticity is well known in a variety of animals (e.g. Gross 1996; Oliveira et al. 2008) including fruit flies (*D. melanogaster*), in which the mating duration of males previously housed with one or more males is longer than that of males housed alone (Bretman et al. 2010).

Although it is clear that the social environment of male fruit flies strongly affect their behaviour, we still lack a true understanding of the relevant factors involved, their effects on males' physiology,

brain and behaviour, and whether such effects constitute adaptive phenotypic plasticity (see Dukas 2004a). For example, in *D. pseudoobscura* (Noor 1997), as well as *D. persimilis* and *D. melanogaster* (R. Dukas, unpublished data), males isolated for 1 day prior to the test court females more vigorously than males that remain in single-sex groups. This possibly nonadaptive plasticity might merely reflect the fact that males housed with only males for a few days habituate to their all-male setting and stop approaching other flies. Furthermore, in female choice experiments, male *D. melanogaster* kept in larger per capita space had a 2:1 mating advantage over males kept at smaller per capita space when fly numbers and food availability were carefully controlled. The males of the distinct density treatments, however, showed no detectable behavioural differences, which suggested that female choice was involved (Dukas & Mooers 2003).

In summary, it seems that mature-male fruit flies vigorously court immature males because recently eclosed males do not yet produce the key pheromone that indicates their sexual identity. Although the immature males do not appear to gain from receiving courtship, frequent encounters with mature males cause young males to mature into a more sexually aggressive phenotype than young males raised alone.

## Acknowledgments

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