Learning about prospective mates in male fruit flies: effects of acceptance and rejection

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Neurogenetic research in fruit flies (*Drosophila melanogaster*) has established numerous protocols that illustrate flies' rich behavioural repertoire as well as excellent learning and memory abilities. To place that information in an evolutionary context, we followed up on earlier studies by evaluating the effect of experience with a variety of relevant female categories on males' courtship behaviour. We found, for the first time, that the experience of mating with conspecific females caused males to subsequently show lower levels of courtship towards heterospecific females compared to control males. These courtship levels, however, were higher than those of males that had experienced rejection by heterospecific females. Courtship reduction was selective: males that mated conspecifically did not subsequently show low levels of courting other conspecific females, and males that were rejected by recently mated conspecific females did not show low levels of courting heterospecific females. Interestingly, males that mated with virgin conspecifics and males that were rejected by virgin heterospecifics subsequently showed similar levels of low courtship towards recently mated conspecific females, suggesting that males find mated females unattractive after encountering virgin females. Males, however, showed higher levels of courtship towards mated females after mating with simulated mated females than after mating with control virgin females. Overall, our results indicate that adaptive use of learning by male fruit flies enhances mating success and reduces time and effort pursuing relatively less desirable or attainable females.

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The successful establishment of *Drosophila melanogaster* as a key model system for studying the neurogenetics of learning and memory (Quinn et al. 1974; Tully 1996; Keene & Waddell 2007) has led to the proliferation of ingenious protocols for quantifying learning and memory in this species (Davis 1996; Siwicki & Ladewski 2003). The advanced mechanistic work on fruit fly learning has highlighted two significant issues. First, evolutionary biologists got it wrong by underestimating learning in short-lived, small-brained insects (Mayr 1974). Second, we only have limited knowledge of the function, adaptive significance and evolutionary consequences of fruit fly learning. This is in spite of the fact that fruit flies have also served as a prominent model system in evolutionary biology and that learning can influence key evolutionary processes, including the strength and direction of sexual selection and the fate of partially diverged populations (e.g. Kamo et al. 2002; Beltman & Metz 2005; Lynn et al. 2005; Servedio et al. 2009).

To help fill the gaps in our knowledge of the adaptive significance of learning in fruit flies, we have conducted a series of studies over the past decade to assess the adaptive function of learning in the context of sexual behaviour in fruit flies. Briefly, we have found that male fruit flies can quickly learn to reduce courtship selectively towards female classes that reject them, including heterospecific and recently mated females (Dukas 2004, 2005, 2009), and that such learning can reduce the frequency of matings between partially diverged populations (Dukas 2008; Kujtan & Dukas 2009). Our theoretical analyses identified the key ecological parameters that would make males' learning about females most beneficial. These included high encounter rates with females, high frequencies of sexually receptive females and high rates of acceptance by sexually receptive females (Dukas et al. 2006).

Our models also indicated that males learn most from acceptance by receptive females and least by rejection. This appeared sensible because sexually receptive females may reject males they find unattractive, meaning that both receptive and unreceptive females may reject males but only receptive females accept them. In short, acceptance provides more information than rejection because only the former clearly distinguishes one female class from the others. Following the publication of our models (Dukas et al.

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2006), however, experiments on *Drosophila persimilis* and *Drosophila pseudoobscura* indicated no effect of acceptance by conspecific females on males' courtship of heterospecific females. Specifically, males of both species quickly learned to reduce courtship towards heterospecific females that rejected them, but they failed to reduce heterospecific courtship after mating conspecifically (Dukas 2009). This suggested that our functional understanding of the males' learning (Dukas et al. 2006) was incomplete.

To improve our understanding of the adaptive function of learning in male fruit flies, we tested the effects of a few relevant types of experience not examined previously on learning by the males. Specifically, we first tested whether males reduce heterospecific courtship after mating with conspecific females and compared the magnitude of the effect to the already known effects of rejection by heterospecific females. Second, to assess how specific the effects of experience are, we tested whether rejection by heterospecific females or acceptance by conspecific females affects males' propensity to court recently mated females. Third, we asked whether experiencing rejection by recently mated females reduces males' subsequent courtship of heterospecific or immature females. Finally, we tested whether males that succeeded in mating with simulated recently mated females would subsequently find recently mated females more attractive than would males that had previously mated with virgin females.

GENERAL METHODS

We used Canton-S *D. melanogaster* and *Drosophila simulans* established from wild flies collected in southern California in 2009. These sister species are almost identical in visual appearance but vary slightly in smell and song (Schilcher & Manning 1975; Kawanishi & Watanabe 1980; Jallon & David 1987; Cobb & Jallon 1990). The two species have similar global geographical distributions. Although they commonly occur in sympatry, *D. simulans* is more common farther from human habitations than is *D. melanogaster* (Carracedo & Casares 1985; Ashburner 1989; Gromko & Markow 1993). We kept the flies in large 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.

We collected males and females within 8 h of eclosion and placed them 20 per single-sex standard food vial. We moved the focal males into individual vials 1 day prior to training and test. With the exception of the immature females, we used 4-day-old flies. Immature females were younger than 20 h, and recently mated females were mated when they were 3 days old. The focal males in all experiments were *D. melanogaster*. For clarity, we refer to conspecific females by their category only. 'Conspecific' implies mature virgin females; 'immature females' consisted of sexually attractive females that always rejected males; 'recently mated females', identified by males based on their distinct odour (Ejima et al. 2007), rejected males. Finally, we refer to the single class of mature virgin female *D. simulans* as 'heterospecific females'.

All the experiments involved a training phase followed by a test. In experiment 1, we recorded male courtship during both training and test. In the rest of the experiments, we only observed the training vials, verifying that all males courted, that the males in vials with conspecific mature virgin females mated once, and that the males placed with females that were supposed to reject them did not mate. We always conducted all trial types within an experiment simultaneously to eliminate time confounds. Observers blind to males' experience and female category recorded the males' courtship behaviour. Our main focus was the proportion of time that males spent courting females, but we also examined courtship

latencies, defined as the time elapsed from the start of the trial until the first courtship bout in each vial. Vials with no courtship received the maximum possible latency, which was the trial duration. We conducted statistical analyses on arcsine square-root-transformed proportions and log-transformed durations, which met ANOVAs assumptions.

EXPERIMENT 1: EFFECTS OF HETEROSPECIFIC REJECTION AND CONSPECIFIC ACCEPTANCE ON HETEROSPECIFIC COURTSHIP

We tested whether males that mate with conspecific females subsequently find heterospecific females unattractive. Furthermore, we examined whether males that experience a combination of conspecific matings and heterospecific rejections show stronger avoidance of heterospecific females than males that experience only heterospecific rejections. We compared males in these two treatments to males in the two baselines of heterospecific rejections and to males that had no experience with females. We predicted that the males would show heterospecific courtship (1) least after experiencing both heterospecific rejections and conspecific matings, (2) more after experiencing only heterospecific rejection, (3) even more after experiencing only conspecific matings and (4) even more with no courtship experience.

Methods

The protocol was similar to that described in Dukas (2009). Briefly, all the males received four training sessions each taking place in a new vial. Sessions with heterospecific females and no female lasted 30 min, and sessions with conspecific females ended once the males had finished mating. The training sessions were separated by 15 min breaks, and then, following a final 15 min break, we conducted the 15 min test, in which we placed each male in a vial containing two heterospecific females. All males in the conspecific/heterospecific treatment experienced two trials of conspecific mating and two trials of heterospecific rejection (Table 1). Males in the heterospecific treatment experienced two trials of heterospecific rejection. Males in the conspecific treatment experienced two trials of conspecific mating. The final treatment consisted of males that experienced no females (Table 1). We alternated the order of female presentation so that half of the replicates encountered conspecific females first and half encountered heterospecific females first. Preliminary analyses indicated no order effects ($F_{1,136} = 0.7$, $P = 0.8$), which are not discussed further here.

Analyses

Our original sample size of 144 males was reduced to 143 males owing to a single lost male. For the test data, we calculated for all trials

Table 1
Protocol used in experiment 1

	Male treatment			
	Con/Hetero	Hetero	Con	None
Trial 1	C	–	C	–
Trial 2	H	H	–	–
Trial 3	C	–	C	–
Trial 4	H	H	–	–
Test	H	H	H	H

Male *D. melanogaster* experienced mating with conspecific females (C), rejection by heterospecific females (H), or no female (–) during training trials prior to encountering heterospecific females during the test. For males that experienced conspecific and heterospecific females during trials (Con/Hetero), half the replicates experienced the schedule given in the table, and the other half had the order of presentation reversed, with heterospecific females encountered first.

the proportion of time the males spent courting during the 15 min test duration. We conducted four planned comparisons involving (1) the conspecific/heterospecific versus heterospecific treatments, (2) the conspecific/heterospecific and heterospecific versus conspecific treatments, (3) the conspecific/heterospecific and heterospecific versus none treatments, and (4) the conspecific versus none treatments. A preliminary analysis of the courtship latencies indicated no differences between treatments (ANOVA: $F_{3,139} = 0.1$, $P = 0.95$).

To examine data from the training phase, we calculated for all training trials the proportion of time the males spent courting out of the total time available, which was the 30 min trial duration in all trials with no matings (all heterospecific trials and six (4%) conspecific trials) and the mating latency in all but six of the conspecific trials. We then ran two repeated measures ANOVAs, one for the heterospecific trials of the conspecific/heterospecific and the heterospecific treatments, and the other for the conspecific trials of the conspecific/heterospecific and the conspecific treatments (Table 1).

Results

Test

The type of experience significantly affected the males' heterospecific courtship (ANOVA: $F_{3,139} = 4.3$, $P < 0.01$; Fig. 1). While males of the conspecific/heterospecific and heterospecific treatments spent similar proportions of time courting heterospecific females ($t_{139} = 0.5$, $P = 0.6$) and nonsignificantly less than males of the conspecific treatment ($t_{139} = 1.8$, $P = 0.07$), they courted significantly less than the inexperienced males ($t_{139} = 3.5$, $P < 0.001$). Males of the conspecific treatment courted nonsignificantly less than the inexperienced males ($t_{139} = 1.4$, $P = 0.16$; Fig. 1).

Training

Males of both the conspecific/heterospecific and heterospecific treatments showed a strong reduction in heterospecific courtship from the first to second heterospecific trials (repeated measures ANOVA, within-subject effect: $F_{1,70} = 52.3$, $P < 0.001$; Fig. 2a). There was also a significant treatment by trial interaction ($F_{1,70} = 4.8$, $P < 0.05$), which, unexpectedly, was caused by more courtship in the heterospecific treatment than in conspecific/heterospecific treatment during the first training trial (Fig. 2a). We have no explanation for this result. A further analysis of the conspecific/
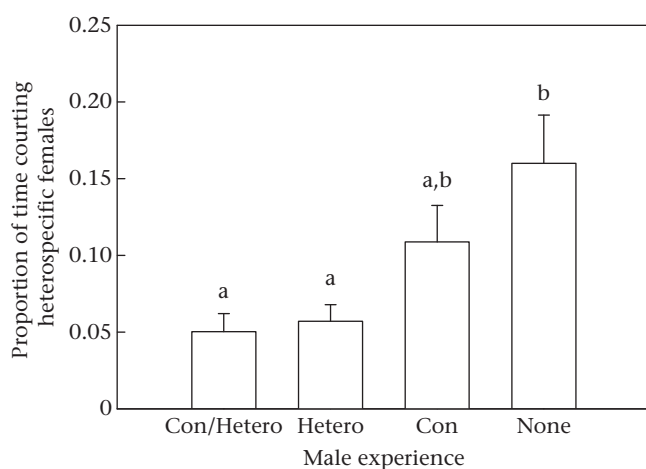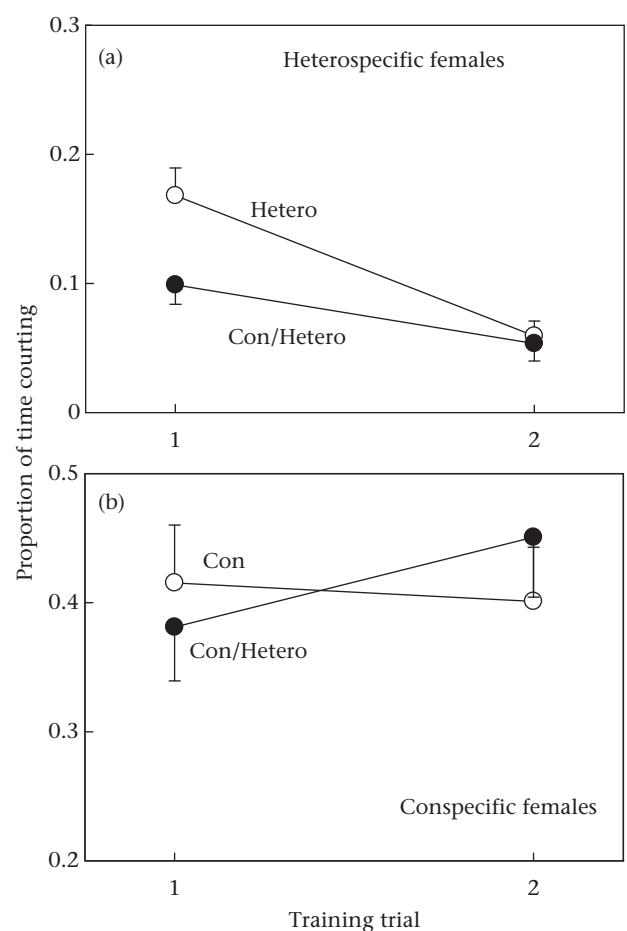
Figure 2. Mean ± SE proportion of time that male *D. melanogaster* ($N = 143$) spent courting (a) heterospecific and (b) conspecific females during training trials of experiment 1 (see protocol in Table 1). Males experienced mating with conspecific females and rejection by heterospecific females (Con/Hetero), rejection by heterospecific females (Hetero), or mating with conspecific females (Con).

heterospecific treatment revealed no trial order effect ($F_{1,34} = 2.1$, $P = 0.15$). Unlike the heterospecific trials, males of both the conspecific/heterospecific treatment and the conspecific treatment showed no reduction in courtship duration from the first to second conspecific trials (within-subject effect: $F_{1,69} = 0.9$, $P = 0.5$; treatment by trial: $F_{1,69} = 0.9$, $P = 0.3$; Fig. 2b).

EXPERIMENT 2: FURTHER TESTS ON THE EFFECT OF CONSPECIFIC ACCEPTANCE ON HETEROSPECIFIC COURTSHIP

The results of experiment 1 were somewhat equivocal as they suggested that experience with conspecific females reduced subsequent heterospecific courtship even though that reduction was not significant (Fig. 1). To assess this possibility critically, we conducted another experiment in which we gave males only 30 min of experience followed by a test. This modification was based on the results from experiment 1, which suggested that the 30 min experience was sufficient (Fig. 2a). The shorter training duration allowed us to test more flies and thus increase our statistical power. As before, we predicted that the males would show the least amount of courting heterospecific females after experiencing heterospecific rejection, more heterospecific courtship after experiencing conspecific matings and even more heterospecific courtship with no courtship experience.

Figure 1. Mean ± SE proportion of time that male *D. melanogaster* ($N = 143$) in each treatment spent courting heterospecific females. Treatments as in Table 1. Distinct letters above bars indicate statistically different values.

Methods

The protocol was similar to that of experiment 1. Here males had only a single period of experience lasting a maximum of 30 min or until the end of mating. Our three types of experience were rejection by heterospecific females, mating with conspecific females and no experience with females. We tested 192 males. A preliminary analysis of the courtship latency data indicated significant differences between the treatments (ANOVA: $F_{2,177} = 6.4$, $P < 0.005$) and post hoc comparisons (Bonferroni with $P < 0.05$) indicated significantly shorter latencies for naïve males (mean $\pm$ SE: 62.8 ± 7.9 s) than for males experienced with heterospecific (122.4 ± 17.9 s) or conspecific (110.1 ± 16.9 s) females, which did not differ significantly from each other.

Results

The type of experience significantly affected the males' heterospecific courtship (ANOVA: $F_{2,189} = 9$, $P < 0.001$; Fig. 3). Males that experienced rejection by heterospecific females courted significantly less than males that experienced conspecific mating ($t_{189} = 2.1$, $P < 0.05$), and males that experienced conspecific mating courted significantly less than naïve males ($t_{189} = 2.2$, $P < 0.05$).

EXPERIMENT 3: EFFECTS OF HETEROSPECIFIC REJECTION AND CONSPECIFIC ACCEPTANCE ON COURTING RECENTLY MATED FEMALES

Experiment 1 indicated that a short experience with heterospecific rejection caused a substantial selective decrease in males' heterospecific, but not conspecific, courtship (Fig. 2). This indicates that males can readily distinguish heterospecific from conspecific females. Yet the experience of mating conspecifically had a weaker effect on males compared to the experience of being rejected by heterospecific females (Fig. 3). These results are somewhat inconsistent with our previous results for *D. melanogaster*, which indicated that males show similar levels of reduced courtship towards recently mated females after experiencing rejection by either unreceptive immature females, which are highly sexually attractive, or recently mated females, which

are sexually unattractive (Figure 3 in Dukas 2005). We interpreted the 2005 results as indicating males' reduced propensity to court females with an unattractive odour after encountering females with a highly attractive odour, but a possible alternative is that these males reduced courtship towards recently mated females because they had experienced rejection by attractive immature females. That is, the immature females confounded positive (attractive odour) and negative (rejection) experiences. To untangle the experiences, we tested the positive effect of conspecific mating and the negative effect of heterospecific rejection on males' subsequent courtship towards recently mated conspecific females. We expected the males to find recently mated females unattractive after mating with virgin females but to find them relatively attractive after experiencing rejection by heterospecific females. That is, we predicted that males would show similar low levels of courtship towards recently mated females after being rejected by recently mated females and after mating with virgin females, and that they would show similar high levels of courtship towards recently mated females after being rejected by heterospecific females and when they had no prior experience with females.

Methods

The overall protocol was similar to that of experiment 1 and our previous work (e.g. Dukas 2010). Briefly, the males received 30 min training followed by a 15 min break and a 15 min test. The four training types consisted of two recently mated females, two mature virgin females, two heterospecific females, and no females. We tested all males with mated females.

Analyses

We conducted 288 trials but omitted two males that mated with recently mated females during the test. We compared the proportion of time the males spent courting during the 15 min test using ANOVA with post hoc Tukey pairwise comparisons. A preliminary analysis of the courtship latency data indicated no differences between treatments (ANOVA: $F_{3,282} = 1.8$, $P = 0.15$).

Results

The type of experience significantly affected the proportion of time that males spent courting recently mated females (ANOVA: $F_{3,282} = 4.6$, $P < 0.005$; Fig. 4). Males that experienced conspecific mating and heterospecific rejection showed lower courtship than inexperienced males (Tukey post hoc comparisons: $P < 0.05$) and similar courtship to males that experienced recently mated females (Tukey post hoc comparisons: $P = 1$; Fig. 4).

EXPERIMENT 4: EFFECTS OF REJECTION BY RECENTLY MATED FEMALES ON COURTING HETEROSPECIFIC OR IMMATURE CONSPECIFIC FEMALES

Experiment 3 indicated that both the positive experience of conspecific mating and the negative experience of heterospecific rejection caused males to show similar levels of reduced courtship towards recently mated conspecific females. While the effect of positive experience is in agreement with our theoretical predictions (Dukas et al. 2006), the effect of heterospecific rejection is puzzling. To examine this issue further, we tested the effect of rejection by recently mated females on males' subsequent courtship towards heterospecific and immature conspecific females. We expected no reduced courtship towards either female category after males' rejection by recently mated females.
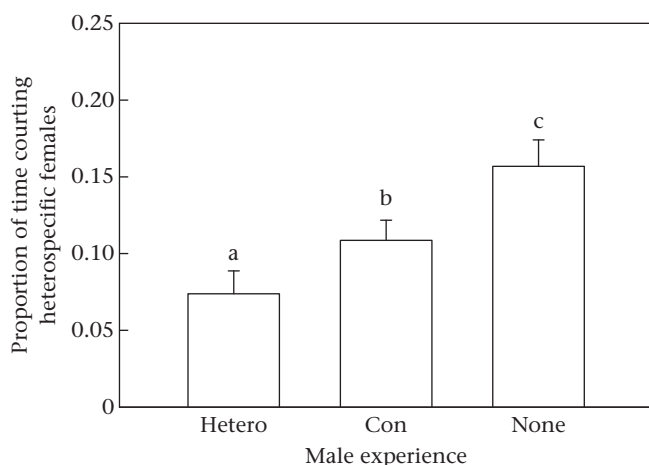
Figure 3. Mean $\pm$ SE proportion of time that male *D. melanogaster* ($N = 192$) with distinct experiences spent courting heterospecific females. Males experienced rejection by heterospecific females (Hetero), mating with conspecific virgin females (Con), or encountered no females (None).
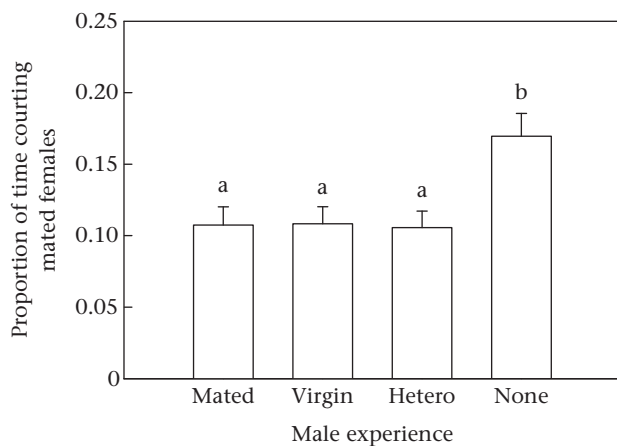
Figure 4. Mean ± SE proportion of time that male *D. melanogaster* (*N* = 286) with distinct experiences spent courting recently mated females. Males experienced rejection by recently mated females (Mated), mating with conspecific virgin females (Virgin), rejection by heterospecific females (Hetero), or encountered no females (None).

Methods

The general protocol was similar to that of experiment 2. Here, two treatments involved training with mated or heterospecific females, followed by a test with heterospecific females, and the other two treatments involved training with mated or immature females, followed by a test with immature females.

Analyses

We conducted 256 trials and performed two analyses. One analysis compared courtship behaviour of males that were tested with heterospecific females after experiencing recently mated or heterospecific females. The second analysis compared males that were tested with immature females after experiencing recently mated or immature females. In addition to the proportion of time spent courting and courting latency, we also calculated courtship persistence, defined as the proportion of time that males spent courting during the period starting with the first courtship bout and terminating at the end of each trial. That is, courtship persistence indicated each male's determination in pursuing the female. Vials with no courtship were treated as missing values.

Results

In the test with heterospecific females, males experienced with recently mated females spent longer proportions of time courting heterospecific females than did males experienced with heterospecific females (ANOVA: $F_{1,124} = 4.3$, $P < 0.05$; Fig. 5a). In the test with immature females, males experienced with recently mated females spent shorter proportions of time courting immature females than did males experienced with immature females (ANOVA: $F_{1,124} = 4.4$, $P < 0.05$; Fig. 5a). Because the latter result was contrary to our prediction, we further explored the males' behaviour. Analyses of courtship latencies revealed that, among the males tested with immature females, the males experienced with recently mated females initiated courtship later than the males experienced with immature females (ANOVA: $F_{1,124} = 10$, $P < 0.005$; Fig. 5b). There was no parallel effect of experience in the males tested with heterospecific females (ANOVA: $F_{1,124} = 0.08$, $P = 0.77$; Fig. 5b). Finally, the males tested with immature females showed similar courtship persistence regardless of their experience (ANOVA: $F_{1,117} = 0.01$, $P = 0.9$; Fig. 5c) whereas, among the males tested with heterospecific females, the males experienced with recently mated
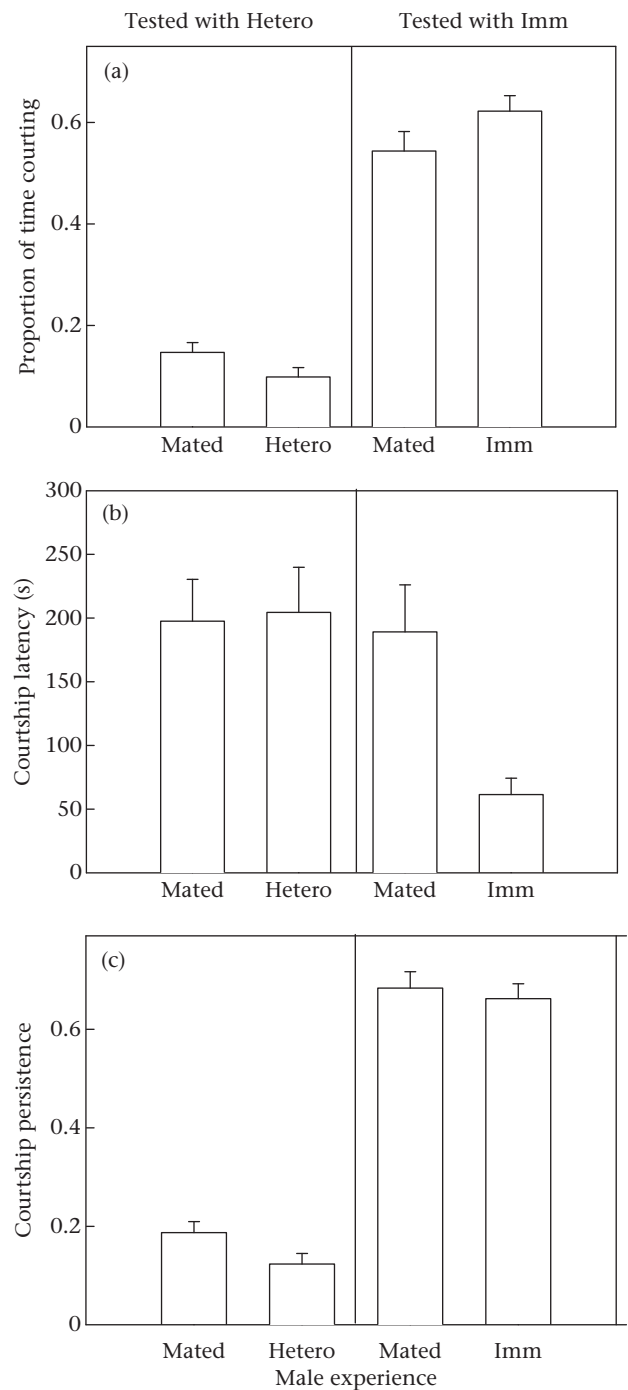
Figure 5. (a) Mean ± SE proportion of time that male *D. melanogaster* spent courting females. (b) Mean ± SE courtship latency from the start of each trial until the first courtship bout. (c) Mean ± SE courtship persistence (proportion of time that males spent courting during the interval from the first courtship until the end of the trial). Males (*N* = 256) were either tested with heterospecific females after experiencing rejection by recently mated females (Mated) or heterospecific females (Hetero), or tested with immature females after experiencing rejection by recently mated females or immature females (Imm).

females showed greater courtship persistence than the males experienced with heterospecific females (ANOVA: $F_{1,113} = 5.5$, $P < 0.05$; Fig. 5c). That is, we could attribute the difference in time spent courting immature females, but not heterospecific females (Fig. 5a), to variation in courtship latency (Fig. 5b), as indicated by courtship persistence (Fig. 5c).

EXPERIMENT 5: EFFECTS OF ACCEPTANCE BY SIMULATED MATED FEMALES ON COURTING RECENTLY MATED FEMALES

The results of experiment 3 (Fig. 4) and previous work (Dukas 2005) indicate that males with any kind of experience subsequently show low levels of courting recently mated females compared with inexperienced males. The experiences include mating with virgin females and rejections by heterospecific or recently mated females. Such male behaviour may be adaptive. On one hand, males that have not encountered females may be willing to court recently mated females even though they are unattractive. Although the probability of mating is lower for mated females than for virgin females, mated females may be the only females currently available. Furthermore, the males' probability of mating with mated females is unknown owing to variation in time and space. Persistent rejection by the recently mated females causes males to reduce courtship of such females. On the other hand, males that have recently encountered virgin females may subsequently devote little courtship effort to recently mated females because they should maximize the time they spend pursuing virgin females in the vicinity, with whom they are more likely to mate. This explanation assumes that the males perceive the closely related heterospecific females as virgin conspecifics. Such an assumption is realistic given that naïve *D. melanogaster* males may spend equal proportions of time courting virgin *D. melanogaster* and *D. simulans* females (Dukas 2004).

To substantiate the adaptive explanation, it would be useful if we can find a scenario where males with courtship experience do not show low levels of courting recently mated females. Males that succeed in mating with recently mated females should not subsequently show low levels of courting such females. The major odour difference between virgin and recently mated females is that recently mated females possess cis vaccenyl acetate (cVA), which is transferred by males during mating (Ejima et al. 2007). Thus, in experiment 5, we applied synthetic cVA to virgin females and tested the effects of mating with such females on males' subsequent courtship towards recently mated females. We predicted that males mated with simulated recently mated females would subsequently show higher levels of courting recently mated females than would males mated with control virgin females.

Methods

We obtained synthetic cVA from Cayman Chemical and diluted it in pure ethanol. In a preliminary experiment, we compared the proportion of time that males spent courting sexually immature females treated with either cVA or ethanol. We applied either 400 ng of cVA diluted in 0.2 μl of ethanol or 0.2 μl of ethanol to the dorsal tip of the abdomen of immature females up to 19 h posteclosion. We tested 63 males.

In the learning experiment, each male experienced either a simulated recently mated female consisting of a mature virgin female treated with 833 ng of cVA diluted in 0.1 ul of ethanol, or a control mature virgin female treated with 0.1 ul of ethanol. We monitored the vials and added a second female of the same treatment into vials in which matings did not occur within about 10 min (48% vials of the cVA treatment and 26% vials of the control treatment: $\chi^2_1 = 6.9, P < 0.01$). The female additions were supposed to compensate for the lower attractiveness of the females treated with cVA. With these female additions, the frequencies of matings were similar between the treatments (67% vials of the cVA treatment and 74% vials of the control treatment: $\chi^2_1 = 0.75, P = 0.4$).

At the end of each mating, we transferred the male into a fresh vial for a break of about 15 min. We then added a female mated earlier in the same morning into the vial and recorded courtship for 15 min. We tested a total of 96 males. Courtship latencies did not differ significantly between treatments (ANOVA: $F_{1,90} = 0.6$, $P = 0.4$), and no male mated during the test.

Results

In the preliminary experiment, males spent significantly lower proportions of time courting immature females treated with cVA than they did immature control females treated with ethanol (ANOVA: $F_{1,58} = 17.5$, $P < 0.001$; Fig. 6a). In the learning experiment, males that had mated with simulated recently mated females spent longer proportions of time courting recently mated females than did males that had mated with virgin females (ANOVA: $F_{1,90} = 6.1, P < 0.02$; Fig. 6b).

DISCUSSION

Experiment 1 indicated that males selectively reduced courtship towards heterospecific females following rejection by such females (Fig. 1). Surprisingly, however, even though males of the conspecific/heterospecific treatment experienced twice the training and a contrast between the distinct categories of accepting and rejecting females, they spent similar proportions of time courting heterospecific females as did males of the heterospecific treatment. We cannot explain this puzzling result, which is similar to the one
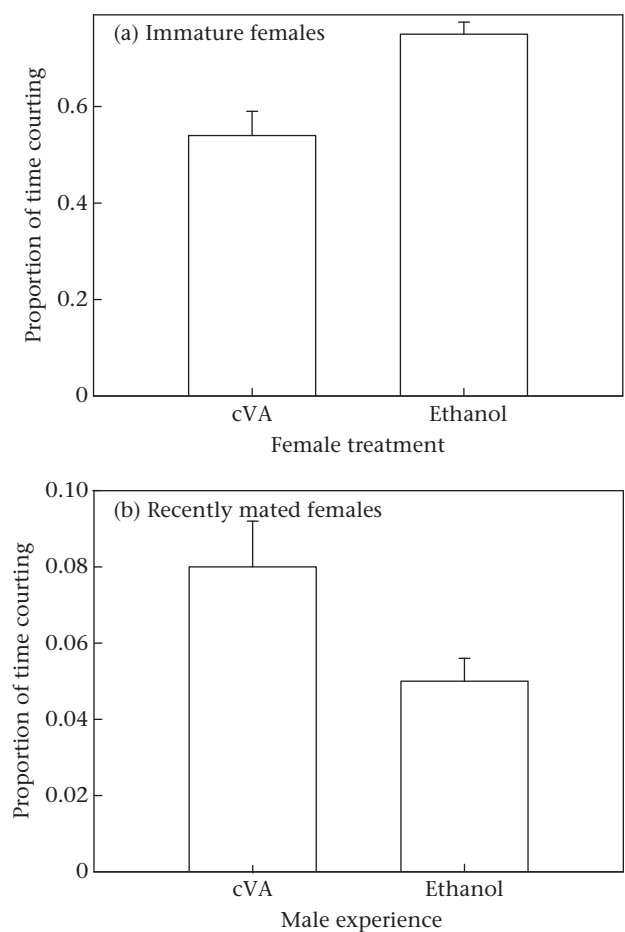
Figure 6. (a) Mean ± SE proportion of time that *D. melanogaster* males (*N* = 63) spent courting immature females treated with cis vaccenyl acetate (cVA) or ethanol. (b) Mean ± SE proportion of time that males (*N* = 96) spent courting recently mated females, which emit cVA received from the males, after mating with conspecific virgin females treated with either cVA (simulated mated females) or ethanol (control).

observed for the species pair *D. persimilis* and *D. pseudoobscura* (Dukas 2009). Males showed significant reduction in courting heterospecific females after a single 30 min trial, as illustrated in the training data (Fig. 2a). Importantly, the training data indicated that males showed neither overall sensitization after mating nor general reduction in courtship following rejection. First, the mating prior to the second heterospecific trial in the conspecific/heterospecific treatment did not increase the males' subsequent heterospecific courtship compared to the heterospecific treatment (Fig. 2a). Second, the rejection before the second conspecific trial in the conspecific/heterospecific treatment did not decrease the males' subsequent conspecific courtship compared to the conspecific treatment (Fig. 2b).

Unlike the clear effects of rejection by heterospecific females, mating with conspecific females did not result in significant changes in the proportion of time that males spent courting heterospecific females (Fig. 1). The suggestive pattern, however, invited a replication. Indeed, in experiment 2, which had a larger sample size, we documented a significant intermediate effect of experience mating with conspecific females on subsequent courtship of heterospecific females (Fig. 3). In general, this result agrees with the Bayesian framework of Dukas et al. (2006). However, our finding that the effect of experience was less with conspecific females than with heterospecific females suggests sensible modifications to that model (Dukas et al. 2006). We previously assumed that males expect to encounter either receptive conspecific females or nonreceptive 'other' females. Given this assumption, males that have mated with conspecific females should subsequently consider all other female categories as the nonreceptive others. Our results, however, suggest that the males expect to encounter multiple classes of both receptive and unreceptive females. With such an assumption, males that mate with a female of one class should remain at least partially receptive to courting other female classes.

In experiment 3, we found that, contrary to our prediction, males that experienced rejection by heterospecific females later showed low levels of courting mated conspecifics (Fig. 4). We now believe that the males perceived the virgin females of the closely related *D. simulans* as conspecifics and thus found the recently mated females unattractive relative to the recently encountered virgin females. This possibility agrees with our previous results indicating that inexperienced *D. melanogaster* males spend equal proportions of time courting virgin *D. melanogaster* and *D. simulans* females (Dukas 2004).

Experiment 3 also indicated that the experience of mating with conspecific females has a different effect on males' subsequent behaviour towards mated and heterospecific females. While mating with conspecific females had a weaker effect than rejection by heterospecific females on males' subsequent courtship towards heterospecific females (Figs 1, 3), mating with conspecific females had a similar effect to that of rejection by mated females on males' subsequent courtship of mated females (Fig. 4). We know that males do not merely reduce courtship after mating because they showed no such reduction in experiment 1 (Fig. 2b), and they show similar courtship reduction after experiencing rejection by sexually attractive but unreceptive immature conspecific females (Dukas 2005). The most likely reason for the differential effect of experience on males' behaviour towards mated conspecifics and virgin heterospecifics is that mated females possess a conspicuous signal, the pheromone cVA, which indicates low receptivity. Fruit flies possess specific receptors for cVA, which is involved in a variety of social interactions (Bartelt et al. 1985; Kurtovic et al. 2007; Datta et al. 2008; Wang & Anderson 2010; Liu et al. 2011). In contrast, inexperienced male *D. melanogaster* most likely perceive the closely related heterospecific *D. simulans* females as conspecifics, given their similarity and the enormous variation that exists in the only

cues known to distinguish them from conspecific females, their cuticular hydrocarbons (Jallon & David 1987; Ferveur et al. 1996).

Experiment 4 showed, in agreement with our prediction, that males experiencing rejection by mated conspecific females were eager to court virgin heterospecific females (Fig. 5a). This result is another indication that males are specific in implementing their experience rather than showing a nonselective reduction in courtship after experiencing rejection. We were surprised, however, that the males showed less courtship towards immature conspecifics after experiencing rejection by mated conspecifics (Fig. 5a). Our further examination of this result suggested a key sensible feature of a future male courtship model. In addition to updating their information about the classes of females that they encounter, court and mate with, males should also assess their probability of encountering receptive females at a given time and location. That is, males that have encountered only unreceptive females for some time may conclude that no receptive females are available and reduce their effort searching for females. On the other hand, males that know that there are unmated females in their vicinity may increase their search effort. Indeed, we found that, compared with males that had previously encountered sexually attractive, but immature, females, males that had previously encountered mated females were slower to initiate courtship in the subsequent test (Fig. 5b). Once this difference in courtship latency was accounted for, the males showed similar persistence in courting immature females, as we had predicted (Fig. 5c).

While we could provide an adaptive explanation for all of our results, we had the inconvenient observation that males' experiences with any kind of female caused a subsequent reduction in the proportion of time they spent courting mated females (Fig. 4). To illuminate this issue, we applied synthetic cVA to virgin females, which made them similar to recently mated females and hence less attractive to males (Fig. 6a). We found, as predicted, that males that had mated with such simulated recently mated females were subsequently more eager to court recently mated females than were males that had mated with control virgin females (Fig. 6b). These results, in addition to strengthening the adaptive interpretation of all of our results, also illustrate the utility of learning in the males. Recently mated females do occasionally accept males. If a recent acceptance by a recently mated female predicts a likely subsequent acceptance by another recently mated female, then a successful male should be more persistent in pursuing such females than a male that has recently mated with a virgin female.

In summary, our results significantly broaden our previous investigation on the adaptive significance of male fruit fly courtship, which places the detailed neurogenetic findings on learning in the context of sexual behaviour in a richer evolutionary context. While the role of learning in the context of sexual behaviour has been widely appreciated in some taxa, most notably birds (Immelmann 1972; West & King 1988; Irwin & Price 1999; ten Cate & Vos 1999; Price 2008), only recently has there been a broader appreciation of the possibility that learning in the context of sexual behaviour is widespread in insects (Bailey & Zuk 2009; Svensson et al. 2010; Fowler-Finn & Rodríguez 2012). Given this realization, we should now focus on the importance of such learning for sexual selection and incipient speciation.

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