Learning magnifies individual variation in heterospecific mating propensity

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Recent research indicating learning in the context of sexual behaviour in fruit flies suggests that learning could increase levels of assortative mating between partially diverged populations. We present a graphic model examining the role of learning and a series of experiments evaluating assumptions and predictions of the model. We found that male *Drosophila persimilis* that previously succeeded in mating with females of the sibling species, *D. pseudoobscura*, did not have a higher heterospecific mating success than males that were either virgin or previously mated with conspecific females. On the other hand, female *D. pseudoobscura* with apparently strict mating criteria, which rejected heterospecific males, were also more likely to reject conspecific males than were females inexperienced with males. Finally, *D. persimilis* males previously rejected by heterospecific females courted significantly less and had half as much heterospecific mating success as males previously accepted by heterospecific females. These results, combined with previous evidence demonstrating that males rejected by heterospecific females learn to avoid courting such females, indicate that learning can increase phenotypic divergence between populations with partial pre-mating isolation.

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The recent surge in research on mechanisms leading to speciation (Coyne & Orr 2004; Grant & Grant 2008; Price 2008) has been accompanied by renewed interest in the role of learning in population divergence (e.g. Lachlan & Servedio 2004; Magurran & Ramnarine 2004; Beltman & Metz 2005; Verzijden & ten Cate 2007; Servedio et al. 2009). New information regarding learning in the context of courtship and mate choice in fruit flies (*Drosophila* spp.) has provided exciting fresh opportunities for examining effects of learning on processes leading to population divergence in one of the key model organisms used in research on speciation (e.g. Coyne & Orr 1989; Noor & Feder 2006). Specifically, work with two pairs of sibling species, *D. melanogaster*–*D. simulans* and *D. persimilis*–*D. pseudoobscura* has indicated that males that experience rejection by heterospecific females rapidly learn to reduce courtship of such females (Dukas 2004b, 2008, 2009).

Whereas the studies indicating learning in the context of sexual behaviour in fruit flies suggested that learning can increase assortative mating, the experimental protocols used actually simulated interactions between two species that are already fully reproductively isolated because the experience phases always included heterospecific rejection. To understand the role of learning in the divergence of populations that are only partially isolated, however, we have to simulate the realistic scenario in which some proportion of the males succeed in acquiring heterospecific mates while the majority fail. Learning could contribute to population divergence even under such realistic settings, and here we examine this possibility using a graphical model and empirical tests.

Suppose that two populations that are partially reproductively isolated come into contact in sympatry and that males of population A encountering females of population B court them as much as they court females of population A. Furthermore, suppose that heritable variation in attractiveness among the males and in permissiveness among the females determine the small fraction of individuals that accomplish interpopulation matings. Figure 1 presents a graphical representation of this scenario following standard signal detection theory (Green 1966; Wiley 1994) as applied explicitly to fruit fly courtship (Dukas et al. 2006). Heritable individual variation in attractiveness and permissiveness is well known in a variety of species including fruit flies (Manning 1967; Carracedo & Casares 1985; Jamart et al. 1993; Andersson 1994; Jennions & Petrie 1997). Such interpopulation interactions would result in a minority of males and females producing hybrids with presumably lower fitness and most males being rejected by females of the other population. These rejected males would learn to avoid females of population B and hence reduce their future probability of heterospecific mating (Fig. 2). That is, learning could magnify the effects of heritable variation and this could facilitate population divergence over time.

To evaluate the above model, we conducted a series of experiments with the sibling species *D. persimilis* and *D. pseudoobscura*.

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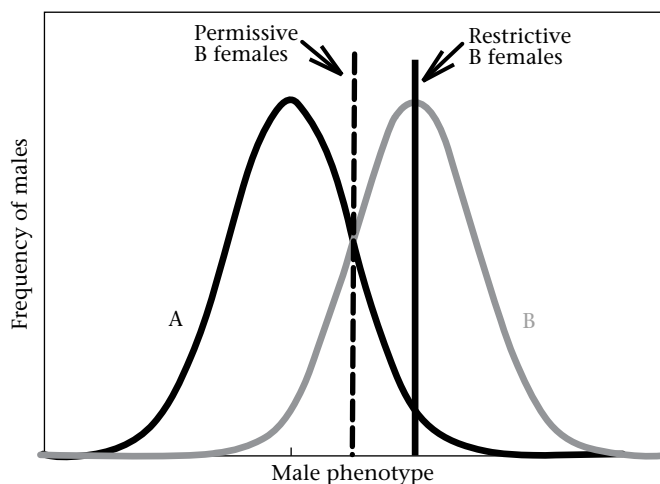
Figure 1. Hypothetical frequency distributions of male phenotypes in two diverging populations, A and B, and two hypothetical mating criteria of females in population B. The *X* axis represents the set of traits determining the perceived phenotype of males by females of population B. The restrictive females are more likely to reject both heterospecific and conspecific males than are the permissive females.

Flies of these recently diverged species look alike and are similar in size, but the males differ slightly in their cuticular hydrocarbons and courtship songs. Heterospecific matings are frequent in the laboratory, especially between naïve pairs of allopatric female *D. pseudoobscura* and male *D. persimilis* (e.g. Mayr 1946; Noor 1995; Dukas 2008). We tested three predictions addressing, respectively, individual variation in male attractiveness to heterospecific females, individual variation in female permissiveness, and whether learning can magnify individual variation in mating success. Specifically, we predicted that, first, males that had previously succeeded in mating with heterospecific females would have a higher heterospecific mating success than either virgin males or males that had previously mated with conspecific females. Second, females that had rejected heterospecific males, which presumably possess a more restrictive mating criterion (Fig. 1), would be more likely to reject conspecific males than randomly chosen virgin females. Finally, males that had been rejected by heterospecific females would show less courtship towards and

Figure 2. Probability of heterospecific mating success by population A males as a function of either their own phenotype or the permissiveness of population B females. Rejection experience changes the phenotype of population A males, which reduces their subsequent probability of heterospecific mating.

obtain fewer matings with such females compared to males that had previously been accepted by heterospecific females.

GENERAL METHODS

We used stocks of *D. persimilis* and *D. pseudoobscura* provided by the *Drosophila* Tucson Stock Center, Tucson, Arizona, U.S.A. *Drosophila pseudoobscura* were initially collected in Tucson, Arizona in 2004, and *D. persimilis* were initially collected on Santa Cruz Island, California, U.S.A. in 2004. Arizona is outside the range of *D. persimilis*, which only occurs along the Pacific Coast of North America, where the two species coexist in sympatry (Dobzhansky & Powell 1975; Markow & O'Grady 2005). The flies were maintained in large population cages housed in distinct environmental chambers and fed standard fly medium.

We collected virgin flies within 8 h of eclosion. The flies were anaesthetized with CO_2, separated by sex, placed in groups of 20 in 40 cm^3 vials, each containing 5 cm^3 of standard fly medium, and kept in the environmental chambers. One day before the start of an experiment, we moved males into individual vials containing standard food medium because such isolation increases male courtship and mating success (Noor 1997).

In all the experiments, the heterospecific pairings involved male *D. persimilis* and female *D. pseudoobscura* because such pairings result in a higher frequency of heterospecific matings than the alternate pairings (Noor 1995). Each experiment consisted of two phases with the second phase being the test in which we monitored matings in all the vials and conducted continuous behavioural observations on a sample of the vials. All observations were conducted by observers blind to fly treatment. We used logistic regression to analyse the mating data and ANOVAs on arcsine square-root transformed courtship proportions. The transformed data met ANOVA assumptions. We attempted to maximize the sample sizes for each experiment but the number of trials varied among experiments owing to insufficient availability of flies and fewer than predicted heterospecific matings.

Experiment 1a: Males Mated with Heterospecific Females versus Virgin Males

Methods

Here we tested for repeatable variation in male heterospecific mating success (Fig. 1). We predicted that *D. persimilis* males that had previously mated with heterospecific females would have a higher heterospecific mating success than virgin *D. persimilis* males. We also predicted that the proportion of time spent courting by males in each of the treatments would be similar because both male treatments should perceive the females as highly desirable mates. Male *D. persimilis* can readily mate conspecifically at least twice within a few minutes (Dukas 2009, unpublished data). We used 6-day-old females and 4-day-old males and conducted 240 trials with 120 males per each of the two conditions. Each male was subjected to two phases detailed below.

Phase 1. Randomly selected males were placed one per vial either alone or with a heterospecific female. The virgin males and the males that completed heterospecific matings were used in phase 2.

Phase 2. At the end of each heterospecific mating, the mated male was placed in a fresh vial along with two virgin heterospecific females. Then a matched virgin male from phase 1 was also placed in a fresh vial along with two virgin heterospecific females. All vials were monitored for 30 min and mating was recorded using a custom-designed computer program.
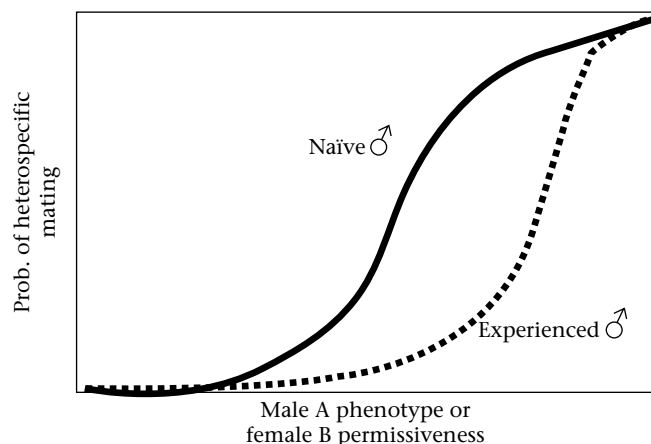
In addition, the courtship behaviour in randomly selected vials was continuously recorded onto a computer for the first 15 min. Courtship behaviour included following of the female, wing vibrations and attempts to mount the female by the male. For each male, we calculated the proportion of time spent courting out of the total time available, which was the total observation time in all trials with no matings and the mating latency in all trials that ended with matings (e.g. Dukas 2005).

Results

Males that succeeded in mating with heterospecific females in phase 1 were as likely to mate with heterospecific females in phase 2 as were virgin males (logistic regression: Wald test: $\chi^2_1 = 0.08$, $P = 0.8$; Fig. 3a). The two male treatments also spent similar proportions of time courting females ($F_{1,60} = 0.36$, $P = 0.5$; Fig. 3b).

Experiment 1b: Males Mated with Heterospecific versus Conspecific Females

Methods

Female *D. melanogaster* may prefer virgin over recently mated males (Markow et al. 1978) and this could counteract the potential mating advantage of males previously mated with heterospecific females. To eliminate this possible confound, in experiment 1b, we compared the mating success of males previously mated with heterospecific females and males previously mated with conspecific females. We predicted that the males that had previously mated with heterospecific females would have a higher mating success with heterospecific females than the males that had previously mated with conspecific females.

The experiment was similar to experiment 1a except that, in phase 1, we placed randomly assigned males each with either a heterospecific female or a conspecific female. Vials of each treatment in which mating was completed were used in phase 2. We completed 292 trials, 146 per treatment.

Results

Males that had mated with heterospecific females in phase 1 were as likely to mate with heterospecific females in phase 2 as were males that had mated with conspecific females in phase 1 (logistic regression: Wald test: $\chi^2_1 = 0.592$, $P = 0.442$; Fig. 4a). The two male treatments also spent similar proportions of time courting females ($F_{1,163} = 1.6$, $P = 0.2$; Fig. 4b).

Experiment 2: Rejecting Females versus Naïve Females

Methods

Here we examined individual variation in permissiveness among females (Fig. 1). We predicted that females that had previously rejected heterospecific males would be more likely to reject conspecific males than females that had not encountered males. Randomly chosen females were either given the opportunity to mate heterospecifically or placed alone in vials. Females that had rejected heterospecific males and females that had no experience with males were subsequently tested with conspecific males. The
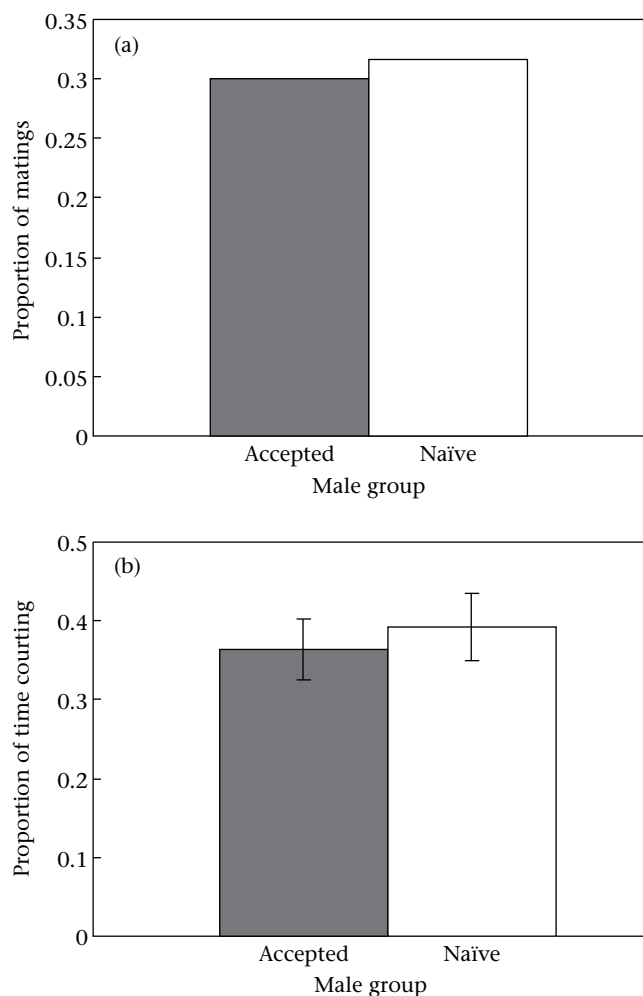
Figure 3. (a) Heterospecific mating success of males that either had been previously accepted by heterospecific females (■) or had no experience with females (□) ($N = 240$), and (b) the corresponding mean (± 1 SE) proportion of time that a random subset of these males spent courting females ($N = 60$).
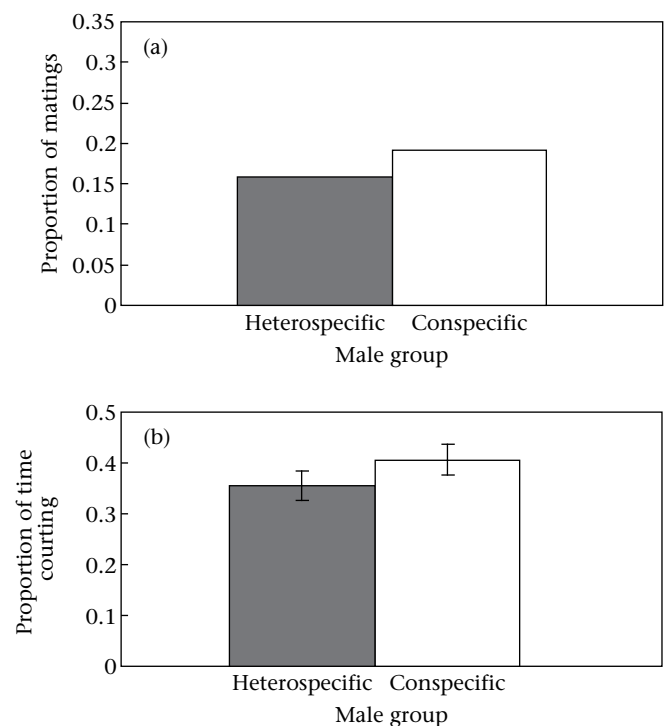
Figure 4. (a) Heterospecific mating success of males that had previously mated with either a heterospecific (■) or conspecific (□) female ($N = 292$), and (b) the corresponding mean (± 1 SE) proportion of time a random subset of these males spent courting females ($N = 164$).

experiment consisted of 452 trials, 226 per each of the two female treatments.

Results

Females that had previously rejected heterospecific males were more likely to reject conspecific males than females that had not encountered males (logistic regression: Wald test: $\chi_1^2 = 4.5$, $P = 0.03$; Fig. 5a). As expected, the conspecific males spent similar proportions of time courting females of the two treatments ($F_{1,54} = 0.09$, $P = 0.8$; Fig. 5b).

Experiment 3a: Males Accepted or Rejected by Heterospecific Females

Methods

Here we examined whether male experience could magnify individual variation in either male mating success or female permissiveness (Fig. 2). We predicted that males that had been previously rejected by heterospecific females would court less and have lower mating success with heterospecific females than would males that had previously succeeded in mating with heterospecific females. Here, phase 1 involved all males placed each with a heterospecific female. In phase 2, we used males that had succeeded in heterospecific matings and males that had courted but failed to mate. We tested a total of 280 males, 140 in each treatment.

Results

Males that had been previously rejected by heterospecific females had significantly lower heterospecific mating success in phase 2 than males that had previously succeeded in mating with heterospecific females (logistic regression: Wald test: $\chi_1^2 = 6.8$,

$P = 0.009$; Fig. 6a). Previously rejected males also spent significantly less time courting heterospecific females compared to previously accepted males ($F_{1,119} = 21.8$, $P < 0.001$; Fig. 6b).

Experiment 3b: Males Accepted by Heterospecific Females versus Rejected by Heterospecific Females and Mated with Conspecific Females

Methods

This follow-up to experiment 3a was conducted to verify that the males that had been rejected by heterospecific females were not substandard males that would be rejected even by conspecific females. Furthermore, by having all males mating prior to the test, we could eliminate a possible effect of short-term sensitization (Broughton et al. 2003; Dukas 2005), which, in experiment 3a, could increase the courtship intensity of the previously accepted but not rejected males. The experiment was similar to experiment 3a except that the males that had been rejected by heterospecific females were allowed to mate with a conspecific female and only the males that completed conspecific mating were tested against the males that mated with heterospecific females. We conducted a total of 520 trials, 260 per treatment.
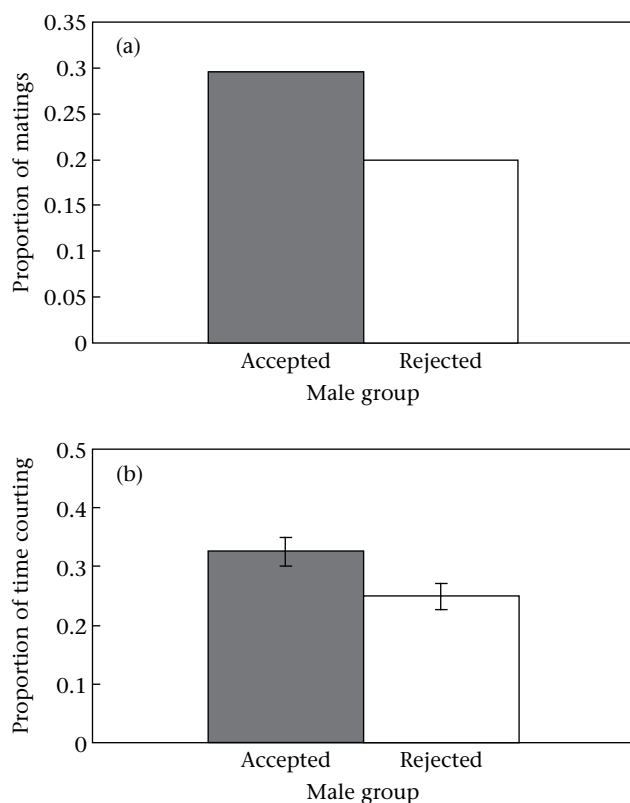
Results

Males that had been previously rejected by heterospecific females and mated with conspecific females had significantly lower mating success in the test phase than males that had been previously accepted by heterospecific females (logistic regression: Wald test: $\chi_1^2 = 6.4$, $P = 0.012$; Fig. 7a). Males that had been previously rejected heterospecifically also spent less time courting
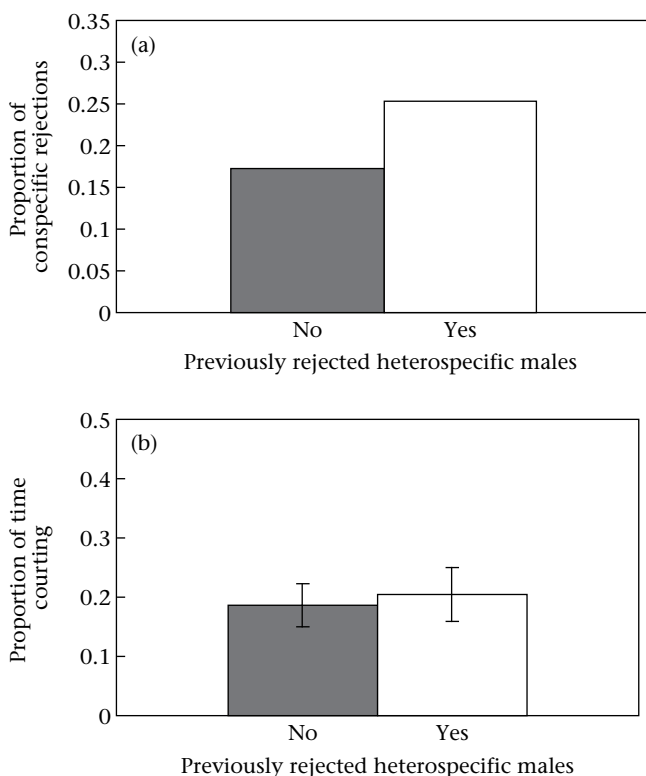
Figure 5. (a) Proportion of conspecific rejections by females with no experience with males (■) and females that had previously rejected heterospecific males (□) ($N = 452$), and (b) the mean proportion of time (± 1 SE) that the conspecific males spent courting each female category ($N = 55$).
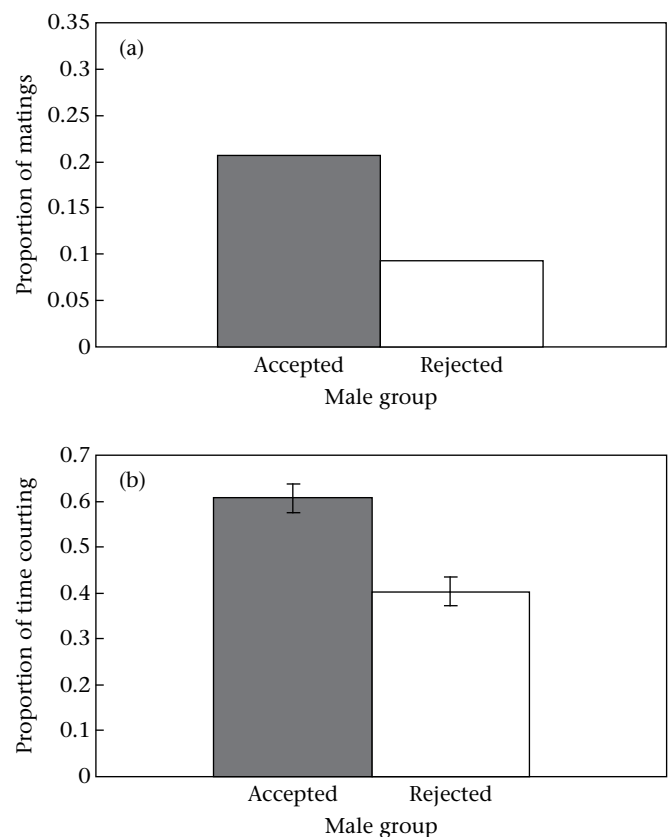
Figure 6. (a) Heterospecific mating success of males that had been either previously accepted (■) or previously rejected (□) by heterospecific females ($N = 280$), and (b) the corresponding mean (± 1 SE) proportion of time that these males spent courting females ($N = 120$).

Figure 7. (a) Heterospecific mating success of males that had been either previously accepted (■) or previously rejected (□) by heterospecific females and accepted by conspecific females ($N = 520$), and (b) the corresponding mean (± 1 SE) proportion of time that these males spent courting females ($N = 172$).

heterospecific females compared to males that had been previously accepted heterospecifically ($F_{1,171} = 5.3$, $P = 0.02$; Fig. 7b).

DISCUSSION

Drosophila persimilis males that had previously succeeded in mating with *D. pseudoobscura* females did not have a higher heterospecific mating success than *D. persimilis* males that were either virgin or that had previously mated with conspecific females (Figs 3, 4). These results indicate that natural variation among the males did not translate into predictable repeatability in heterospecific mating success. Alternatively, our laboratory populations may have not contained sufficient individual variation. On the other hand, the results agreed with our second prediction regarding repeatable female permissiveness (Fig. 1) because restrictive females that rejected heterospecific males were also more likely to reject conspecific males (Fig. 5). We could not test for repeatability of mate choice in the females because the rate of remating was too low even 5 days after the first mating. In summary, our results agree with the prediction that males' initial heterospecific mating success is determined by female variation in permissiveness but not in male phenotype (see the X axis of Fig. 2).

The natural variation among males and females, which initially determined males' probability of being rejected by heterospecific females, dramatically affected males' subsequent probability of achieving heterospecific mating (Figs 6a, 7a). The simplest explanation for this result, that this merely reflected repeatability of mate preference by the females, can be readily rejected because no repeatability in male mating success was evident in experiments 1a and 1b (Figs 3a, 4a). Another alternative, that males that were more

persistent at courting achieved higher heterospecific mating success, can also be rejected because no such pattern was observed in experiments 1a and 1b (Figs 3b, 4b). On the other hand, the possibility that males that were rejected by heterospecific females reduced subsequent courtship of such females is in strong agreement with the evidence. First, in experiments 3a and 3b, the previously rejected males courted females much less than the previously accepted males (Figs 6b, 7b). Second, two previous studies critically tested for learning in male *D. persimilis* by providing all males with a uniform experience of rejection by heterospecific females. Under these controlled experimental settings, the rejection experience caused subsequent selective reduction in males' courtship of heterospecific females (Dukas 2008, 2009). A similar pattern of males selectively reducing courtship of heterospecific females after experiencing rejection by those females has also been documented in the sibling species *D. melanogaster–D. simulans* (Dukas 2004b). Whereas we do not know how male *D. persimilis* learned to reduce heterospecific courtship, extensive research on *D. melanogaster* indicates that the males learn to suppress courtship of unreceptive classes of females identified by their particular blend of cuticular hydrocarbons (Ejima et al. 2005).

Whereas we focus here on learning, the males' experience could also alter their pheromonal profile. This may occur either via direct transfer between individuals (Scott et al. 1988; Ejima et al. 2007; Yew et al. 2008) or through changes in pheromonal production and expression (Moore et al. 1995; Dukas & Mooers 2003; Kent et al. 2008; Krupp et al. 2008). Our experiments effectively controlled for this and other alternatives involving physiological and subtle behavioural changes. First, one would predict that heterospecific mating success would increase subsequent heterospecific mating success if mating success causes the males to express a pheromonal profile more attractive to heterospecific females. In experiments 1a and 1b, however, we found that heterospecific mating success did not increase subsequent heterospecific mating success (Figs 3a, 4a). Second, in experiments 3a and 3b, we found a strong link between experience with heterospecific mating and subsequent courtship duration (Figs 6b, 7b). Such association was also documented in previous experiments (Dukas 2004b, 2005, 2008, 2009). That is, we have clear evidence for a change in behaviour with experience that cannot be readily explained merely by pheromonal changes in the males. Nevertheless, male experience in this system may also influence pheromonal expression, and this possibility requires close examination.

The combination of individual variation in males and females, which determines the outcomes of heterospecific interactions (Fig. 1) and reduction in heterospecific courtship by males previously rejected by heterospecific females (Fig. 2) resulted in the previously rejected males having about half the heterospecific mating success as the previously successful males (Figs 6a, 7a). These results support our proposal that learning can magnify the phenotypic divergence between populations that show partial premating isolation. That is, the initial phenotypic overlap between males of populations A and B in Fig. 1 can be significantly reduced with males' experience of rejection by females from a partially diverged population (Fig. 2). The reduction in effective phenotypic overlap between the populations could lead to increased reproductive isolation over time if the populations reduce gene exchange and continue to diverge into distinct niches. It is widely agreed, however, that learning and phenotypic plasticity can also have the opposite outcome of shielding such populations from divergent selection (Robinson & Dukas 1999; Huey et al. 2003; Price et al. 2003; Dukas 2004a; Servedio et al. 2009). Detailed explicit population genetic models as well as further experiments will be necessary for examining the range of conditions under which learning could indeed enhance incipient speciation.

Acknowledgments

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