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Dynamics of learning in the context of courtship in *Drosophila persimilis* and *D. pseudoobscura*

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Recent data indicating that male fruit flies adaptively reduce courtship of heterospecific females, which typically reject them, suggest that learning could contribute to reduced levels of matings between individuals from diverging populations with partial premating isolation. To further examine the robustness of learning in the context of courtship in fruit flies, I wished to broaden the types of experience provided to males prior to testing. In both *Drosophila persimilis* and *D. pseudoobscura*, alternating trials of mating with conspecific females and rejection by heterospecific females produced the strongest reduction in heterospecific courtship. Trials of rejection by heterospecific females produced equally strong reduction in heterospecific courtship in *D. persimilis* but not in *D. pseudoobscura*, whereas trials of mating with conspecific females did not reduce heterospecific courtship at all. The pattern of strong reduction in heterospecific courtship was also replicated when I simulated the likely natural scenario in which males interact with conspecific females since eclosion and later encounter and experience rejection by heterospecific females. The results indicate that a variety of relevant experiences cause a rapid decrease in the time that male fruit flies spend courting heterospecific females. Such learning in partially reproductively isolated populations could contribute to speciation.

There has recently been a resurgence in research on the mechanisms leading to speciation (Schluter 2000; Coyne & Orr 2004; Noor & Feder 2006; Grant & Grant 2008; Price 2008). While much of the current effort involves work at the genetic and ecological levels, there has also been renewed interest in the effects of learning on population divergence. The diverse theoretical work clearly indicates that learning could be an important factor contributing to speciation (Lachlan & Servedio 2004; Beltman & Metz 2005; Verzijden et al. 2005; Servedio et al., in press). The empirical research, however, is somewhat lagging. Sexual imprinting in birds has been a prime target of research linking learning to speciation (Grant & Grant 1996; Irwin & Price 1999; Slagsvold et al. 2002; Qvarnstrom et al. 2004; ten Cate et al. 2006). However, with the exception of the unusual brood parasitic indigobirds (*Vidua* spp.) (Payne et al. 2000; Sorenson et al. 2003), no study has explicitly linked learning to speciation in birds. Other taxa with limited data suggesting a role of learning in speciation include mammals (Kendrick et al. 1998), fish (Magurran & Ramnarine 2004, 2005; Verzijden & ten Cate 2007) and spiders (Hebets 2003).

Recently, fruit flies (*Drosophila* spp.) have been added to the list of taxa in which learning may have contributed to incipient speciation. First, I documented that male and female fruit flies (*D. melanogaster*) rely on learning to adaptively modify courtship (Dukas 2004, 2005b) and mate choice (Dukas 2005a). Second, in the sibling species, *D. persimilis* and *D. pseudoobscura*, males learned to decrease heterospecific courtship and this resulted in lower levels of heterospecific mating (Dukas 2008). The findings linking learning to increased levels of assortative courtship and mating are intriguing because fruit flies have been a leading model system in research on speciation (Coyne & Orr 2004; Mallet 2006; Noor & Feder 2006). Moreover, unlike birds and fish, large populations of fruit flies can readily be maintained in the laboratory for the necessary critical experiments linking learning to population divergence. First, however, one has to establish whether learning in the context of assortative courtship and mating is a sufficiently robust phenomenon to influence incipient speciation.

In all my previous experiments with male fruit flies mentioned above, I used an avoidance learning protocol in which males previously inexperienced with females were allowed to court and experience rejection by heterospecific females and were later tested with either heterospecific or conspecific females. The selective reduction in heterospecific but not conspecific courtship in experienced compared to inexperienced males indicated learning. Although successful in critically testing for learning, the avoidance learning protocol is unrealistic because it implies that mature males never encounter conspecific females prior to

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encountering only heterospecific females. Hence I wished to enrich the types of experience available to males prior to testing to examine the strength of learning in the context of courtship. Specifically, I predicted that mating with conspecific females and rejection by heterospecific females would result in stronger heterospecific avoidance than only rejection by heterospecific females, and that these two treatments would produce stronger heterospecific avoidance than either only acceptance by conspecific females or no experience with any female, with the latter two treatments showing no heterospecific avoidance. I also expected a decline in courtship duration in successive training trials with heterospecific females but no change in courtship duration in successive training trials with conspecific females. Finally, I expected that males that spent a few days with conspecific females before encountering heterospecific females would also show a strong reduction in heterospecific courtship with experience.

GENERAL METHODS

I used stocks of *D. persimilis* and *D. pseudoobscura* provided by the *Drosophila* Tucson Stock Center (Tucson, Arizona, U.S.A.). The *D. pseudoobscura* population was initiated from flies collected in Tucson, Arizona in 2004. The *D. persimilis* population originated from flies collected on Santa Cruz Island, CA, U.S.A., in 2004. The *D. pseudoobscura* population I used is from outside the geographical range of *D. persimilis*, which only occurs in sympatry with *D. pseudoobscura* along the Pacific Coast (Dobzhansky & Powell 1975; Markow & O'Grady 2005). The flies were maintained in my laboratory since spring 2006 in large cages containing standard fly media kept inside distinct environmental chambers. Flies used in the experiments were sexed within 8 h of eclosion and the females were placed in groups of 20 in single-sex vials. In experiments 1 and 2, males were also placed 20 per vial and transferred into individual vials 1 day before the test because such isolation increases their courtship intensity and mating success (Noor 1997; R. Dukas, unpublished data). Flies in experiments 1 and 2 were 4 days post eclosion. Fly handling and ages in experiment 3 are detailed below. All flies were used only once.

Members of the closely related species pair, *D. persimilis* and *D. pseudoobscura*, are visually indistinguishable but differ in their cuticular hydrocarbons and male courtship song. Inexperienced males of the two species indiscriminately court hetero- and conspecific females but the females prefer to mate with conspecific males. In the laboratory, heterospecific mating is more frequent between male *D. persimilis* and female *D. pseudoobscura* than between the alternate pairing. The hybrid daughters are fertile whereas hybrid sons are infertile (Mayr 1946; Noor 1995; Machado et al. 2002; Ortiz-Barrientos et al. 2004; Dukas 2008). Heterospecific courting is costly for males because they waste time and energy courting females that typically reject them, and infrequent matings produce only half as many fertile offspring. Hence, learning in the context of sexual behaviour could be adaptive in male *D. persimilis* and *D. pseudoobscura*.

EXPERIMENT 1: *D. PSEUDOOBSCURA*

Methods

Training

Males were randomly assigned to each of four treatments, positive/negative, negative, positive and none (Table 1). All males placed with females were observed continuously and the courtship activity of some of the males was recorded for the duration of the training trials (see below). The positive trials involved the completion of mating with a conspecific female. I verified that all males mated during these trials and replaced females in several vials where matings did not occur within 10 min. The trials were terminated upon the completion of mating and were followed by a 1 h break. This long break was designed to diminish effects of sensitization following mating, which are associated with a short-term indiscriminate increase in males' courtship activity (Broughton et al. 2003; Dukas 2005b). The negative trials involved courtship and rejection by heterospecific females, but I included in the analyses the four males that each mated heterospecifically once. Removing these males from the analyses did not alter the results. The negative trials lasted 30 min and were followed by 10 min breaks. Males of all treatments were transferred to new vials at the start of each trial and placed next to each other in the same vial rack to control for the effects of experimenter's handling.

Test

At the end of the break following the last training session, each male was placed with two heterospecific females and males' courtship behaviour was recorded for 15 min as in my previous studies (e.g. Dukas 2004, 2008). Only three males, all belonging to the positive treatment, mated during the test. All the behavioural recordings during training and tests were conducted with observers blind to fly treatment and female species.

Analyses

I trained and tested 128 males, of which I recorded the behaviour of 66 during the training trials. For half the males, the negative trial was first, as in Table 1, and for the other half, the order was reversed. Preliminary analyses indicated no order effects ($F_{1,120} = 1.5$, $P = 0.2$), which are not discussed further here. The main behavioural parameter was the total time spent courting (Dukas & Mooers 2003). I calculated for all trials the proportion of time spent courting out of the total time available, which was the trial duration in all trials with no matings and the mating latency in all conspecific trials and the several heterospecific trials that ended with matings. To test my first set of predictions involving the test phase, I conducted three planned comparisons between (1) the positive/negative versus negative males, (2) the negative versus positive and none males, and (3) the positive versus none males. To test the second set of predictions involving the training phase, I ran two repeated measures ANOVAs, one for the negative trials of the positive/negative and negative males, and the other for the positive trials of the positive/negative and positive males. Analyses were conducted on arcsine-square-root-transformed proportions and log-transformed latency data and the transformed values met ANOVAs assumptions.

Results

Test

The type of experience significantly affected heterospecific courtship by male *D. pseudoobscura* (ANOVA: $F_{3,124} = 7.6$, $P = 0.01$; Fig. 1). The positive/negative males had significantly shorter courtship durations than the negative males ($t_{124} = 3.2$, $P = 0.002$). The negative males did not court significantly less than the positive and none males ($t_{124} = 1$, $P = 0.3$), and there was no significant difference between the positive and none males ($t_{124} = 0.5$, $P = 0.6$).

Training

Both the positive/negative and negative males showed a similar reduction in courtship duration between the first and second heterospecific trials (repeated measures ANOVA: within-subject effects: $F_{1,42} = 40.4$, $P < 0.001$; between-subject effects: $F_{1,42} = 0.02$, $P = 0.9$; Fig. 2a). In contrast, both the positive/negative and positive males showed no reduction in courtship duration between the first