

Species recognition by male swordtails via chemical cues

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Species recognition can often play a key role in female mating preferences. Far less is known about conspecific mate recognition from the male perspective. In many closely related taxa, females exhibit few obvious visual differences and males might have to attend to chemical cues in mate recognition, a possibility that has rarely been explored in vertebrates. Here, we examine male species recognition via odor cues in the swordtail fish, *Xiphophorus birchmanni*. In dichotomous choice experiments we first tested whether males respond to female odor cues. We found that males were attracted to conspecific female odor and those of a related allopatric congener, *Xiphophorus malinche*, over a water control. Males did not, however, respond to the female odor of the more distantly related sympatric platyfish, *Xiphophorus variatus*. We then gave male *X. birchmanni* the choice between conspecific and heterospecific female stimuli. Males, in this scenario, significantly preferred the conspecific odor when the alternative was platyfish. However, when offered odor cues of *X. malinche*, male *X. birchmanni* actually preferred the heterospecific female cue. The complex array of preferences reported here, previously documented only in females, underscores the need to consider the behavior of both sexes in dictating actual mating outcomes. *Key words*: chemical communication, mate choice, Poeciliidae, reproductive isolation, sexual selection, speciation. [*Behav Ecol* 16:818–822 (2005)]

Premating reproductive isolation hinges on the ability of individuals to recognize their mates. While the mating preferences of females have widely been shown to favor conspecifics over heterospecifics (de Kort and ten Cate, 2001; Pfennig, 2000), males have generally been assumed to play a minimal role in mate discrimination. Male behavior, however, can influence matings by reinforcing what females want or, in some instances, by subverting female mating preferences (Wong, 2004; Wong and Candolin, in press). For example, males can be indiscriminate and court both heterospecific and conspecific females alike (Gabor and Ryan, 2001). More insidiously, males can engage in forced or surreptitious fertilization, thereby circumventing female choice (Magurran, 1998). Male mate choice can also be important. Studies have shown, for example, that mate searching and courtship can both impose fitness constraints that limit male mating opportunities, causing males to allocate their mating effort strategically and to discriminate between potential mates (Gowaty et al., 2003; Wong and Jennions, 2003). Although males might be expected to avoid heterospecific females and to prefer conspecifics (Gabor and Ryan, 2001; McKinnon and Liley, 1987; McLennan, 2005; Wiernasz, 1995), there is no a priori reason why male preferences and female cues cannot be decoupled. We know, for instance, that females may sometimes prefer to mate with heterospecific males expressing traits that are more attractive than those found in their own species (Ryan and Wagner, 1987). In a similar way, males could also respond in favor of heterospecific females.

In many taxa, females from closely related species exhibit few obvious visual differences. Chemical cues, however, are often species specific (Wyatt, 2003) and may therefore provide

a mechanism for males to discriminate among potential mates. Despite this, almost all the studies that have examined the role of odor cues in vertebrate mate recognition have looked at the question solely from a female choice perspective (Crapon de Caprona and Ryan, 1990; Hankison and Morris, 2003; McLennan and Ryan, 1997, 1999; but see Cardwell et al., 1992; McKinnon and Liley, 1987; McLennan, 2005; Yambe and Yamazaki, 2001). In this study, we sought to test the role of chemical signals in species recognition by males in a group of closely related fishes with few apparent interspecific differences in female phenotypes (Rauchenberger et al., 1990).

Xiphophorus birchmanni is a small, sexually dimorphic poeciliid fish belonging to the monophyletic northern, or Río Pánuco Basin, clade of swordtails (Morris et al., 2001; Rauchenberger et al., 1990). When in sympatry, *X. birchmanni* hybridizes freely with *Xiphophorus malinche* (Rosenthal et al., 2003), a closely related swordtail (Morris et al., 2001; Rauchenberger et al., 1990), yet there is no evidence of hybridization with the more distantly related platyfish, *Xiphophorus variatus*. Studies focusing on female choice in a range of swordtail species suggest that chemical signals play an especially prominent role in species recognition and mate choice (Crapon de Caprona and Ryan, 1990; Hankison and Morris, 2003; McLennan and Ryan, 1997, 1999). For instance, female swordtails often prefer the visual cues of heterospecifics and yet show robust preferences for conspecific male odor cues (Crapon de Caprona and Ryan, 1990; Hankison and Morris, 2003). In *X. birchmanni*, recent work on female mate recognition demonstrated that females prefer the odor cues of conspecific males over those of male *X. malinche* (Fisher HS, Wong BBM, Rosenthal GG, unpublished data). What about conspecific recognition of females by male *X. birchmanni*? In this study we evaluated whether male *X. birchmanni* attend to species differences in female chemical cues by measuring their responses to odor from female conspecifics and two congeners, *X. malinche* and *X. variatus*.

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Received 1 October 2004; revised 18 March 2005; accepted 21 April 2005.

METHODS

Animal collection and housing

Xiphophorus birchmanni and *X. variatus* were collected from the Río Garces, San Luis Potosí, México (Rauchenberger et al., 1990). *Xiphophorus malinche* were collected from the type locality in the Río Claro at Tlatzintla, a population in allopatry from *X. birchmanni* (Rauchenberger et al., 1990). Sexually mature females were isolated from males for at least 3 weeks prior to testing and were housed in well-planted single-species 40-l aquaria. Animals were maintained on a 12:12 h light:dark cycle and were fed TetraMin flakes, brine shrimp, bloodworms, and mysids. All fish were fed the same diet to control for the possibility that individuals maintained on different diets may elicit different responses (Murray and Jenkins, 1999). Food was delivered in a wash bottle to train test subjects to investigate surface disturbance (see Chemical Preference Tests; McLennan and Ryan, 1997).

Chemical stimulus preparation

We collected female odor cues using 40-l aquaria that were thoroughly cleaned using a solution of soap and hydrogen peroxide. Water in collecting tanks was carbon filtered and aerated for 24 h prior to introduction of females. Female odor stimuli were prepared by placing five sexually mature females of the same species into a single tank (a given stimulus tank, therefore, only contained the odor cues from one species). The mean standard lengths (SL) \pm SD of stimulus females were as follows: *X. birchmanni* = 36.75 \pm 3.37 mm, *X. malinche* = 36.33 \pm 3.08 mm, and *X. variatus* = 36.17 \pm 3.54 mm. The tank housing the females was located adjacent to another aquarium containing two conspecific males to provide them with visual stimulation. After 24 h, females were removed from collection tanks, and stimulus water was used in experiments within 48 h (McLennan and Ryan, 1999). Using this procedure, we obtained stimulus water containing cues from female *X. birchmanni*, *X. malinche*, or *X. variatus*. We also collected stimulus water free from any odor cues by following the same procedure described above but without introducing any fish into collecting tanks.

Chemical preference tests

We used a dichotomous choice setup to assess the response of male *X. birchmanni* to female chemical cues. First, we examined whether males actually respond to the female odor cues collected from conspecifics (*X. birchmanni*) and heterospecifics (*X. malinche*, *X. variatus*) by testing male preference for each female stimulus against a plain water control. Second, we offered males the choice between the female odor of conspecifics and each of the two heterospecifics.

All chemical preference tests were conducted in a thoroughly cleaned aquarium (length \times width \times height = 75 \times 30 \times 30 cm) divided lengthwise into three equal sections (left, right, and center) by lines drawn on the sides of the tank with a waterproof marker and filled with 40 l of filtered, aerated water. Each test tank had two stimulus delivery systems, one at either end of the test tank. Each stimulus delivery system consisted of a 20-l glass aquarium containing stimulus water connected to a 12-mm pipette by 2.5-mm silicone tubing. The pipette was attached to the test tank 2 cm above the water surface, creating a perturbation on the surface when water was dripped into the aquarium.

For each trial, a male *X. birchmanni* (mean SL \pm SD = 36.71 \pm 3.87 mm) was placed into the test tank and allowed to acclimatize for 30 min. Stimulus flow was then initiated simultaneously, dripping water from both stimulus delivery systems

into the tank. Flow was controlled with the use of plastic clamps set at 10 ml/min. We observed focal males behind a screen to minimize disturbance to the fish. The preference test began once the male had passed into both side compartments (left and right) and ran for 10 min. If the male did not visit both compartments within 10 min, the trial was aborted. Association preference for each stimulus in a trial was measured as the time spent by the male in each compartment. Association time is widely used in studies of mate choice in fishes (see Wong, 2004, and references therein) and is the standard measure of mating preferences in poeciliids, including swordtails (Basolo, 1990; Crapon de Caprona and Ryan, 1990; Hankison and Morris, 2002; Kingston et al., 2003; McLennan and Ryan, 1997, 1999; Rosenthal et al., 2002). In a closely related species, *Xiphophorus nigrensis*, association time in laboratory trials is a strong predictor of association in open-field trials and of observed mate choices in the wild (Ryan et al., 1990, 1992). In our study, proximity to the odor source was often accompanied by flexing of the gonopodium, or intromittent organ, a characteristic sexual display (Gabor and Ryan, 2001; Park and Propper, 2002). In the wild, males observed within 1–2 body lengths of females are nearly always performing courtship displays toward those females (Rosenthal, 2000, personal observations). Association time is thus a robust and meaningful estimate of mating preference.

Visual preference tests

In light of the results obtained for the chemical preference trials (see below), we also decided to test the visual preference of male *X. birchmanni* for females of their own species versus those of the closely related congener, *X. malinche*. We followed protocols described in Kingston et al. (2003) and Rosenthal et al. (2002). The experimental setup involved two small aquaria (20 \times 12 \times 12 cm) placed at opposite ends of a larger aquarium (51 \times 28 \times 33 cm) housing the test male. A stimulus female *X. birchmanni* was released randomly into one of the two small aquaria; a size-matched stimulus female *X. malinche* was released into the other. The use of separate containers allowed fish to see one another but prevented the transfer of any odor cues. Fish were acclimatized for 5 min prior to the start of a trial. During this time, a sheet of white cardboard was inserted between the large aquarium and each of the two smaller aquaria to prevent the fish from seeing and interacting with one another. Just before a trial commenced, the white sheets were gently lifted and the amount of time the male spent associating with each female was recorded over a 5-min period. A male was deemed to be associating with a particular female if he was within 10 cm of her aquarium. After the 5-min recording period, the white sheets were reinserted between the aquaria. The females were then switched between the two small aquaria, and after a second 5-min acclimatization period, association time with each female was remeasured for another 5-min recording period. At the end of the trial, we tallied the total time a male spent associating with a particular female during each of the two 5-min recording periods.

All statistical tests are two tailed, and results are presented as mean \pm SE unless indicated otherwise.

RESULTS

Chemical preference tests

Male *X. birchmanni* were attracted to the cues of female *X. birchmanni* (Wilcoxon signed-rank test: $z = 2.39$, $n = 15$, $p = .017$; Figure 1a) and *X. malinche* (Wilcoxon signed-rank test: $z = 2.78$, $n = 15$, $p = .005$; Figure 1b) versus plain water. They did not, however, spend more time with the female *X. variatus*

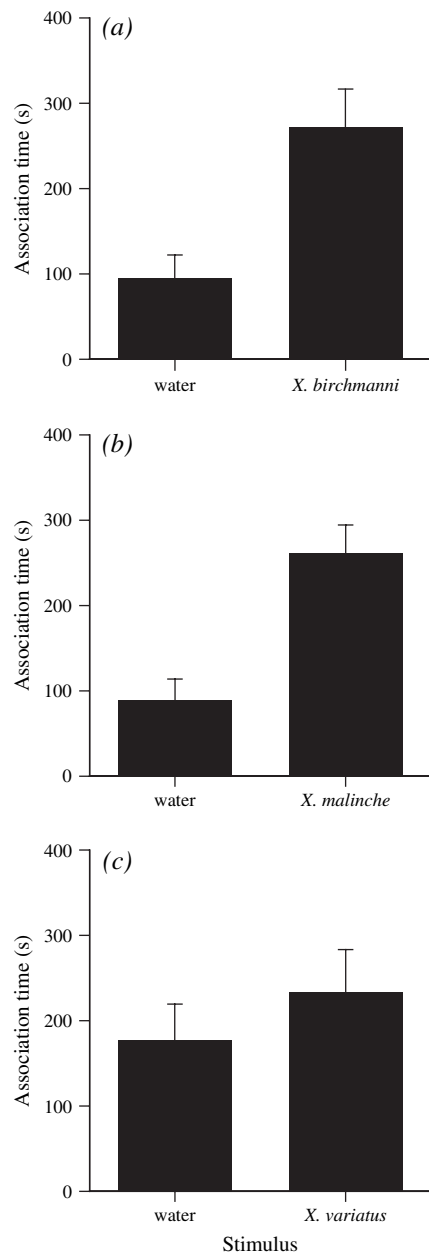


Figure 1 Association time (mean \pm SE) of male *Xiphophorus birchmanni* with odor cues of (a) water versus female conspecific, (b) water versus female *Xiphophorus malinche*, and (c) water versus female *Xiphophorus variatus*.

stimulus over plain water (Wilcoxon signed-rank test: $z = 0.568$, $n = 15$, $p = .57$; Figure 1c).

When offered the choice between the female odor cues of conspecifics and heterospecifics, the amount of time that male *X. birchmanni* spent associating with the conspecific cue depended on which stimulus was offered as the alternative (Mann-Whitney U test: $U = 26$, $n_1 = n_2 = 15$, $p < .001$). We found that male *X. birchmanni* preferred the *X. malinche* stimulus over the odor cues of their own females (Wilcoxon signed-rank test: $z = 1.93$, $n = 15$, $p = .053$; Figure 2a). When given the choice between stimuli from conspecific females and those of *X. variatus*, however, males strongly preferred odors of their own species (Wilcoxon signed-rank test: $z = 2.78$, $n = 15$, $p = .005$; Figure 2b).

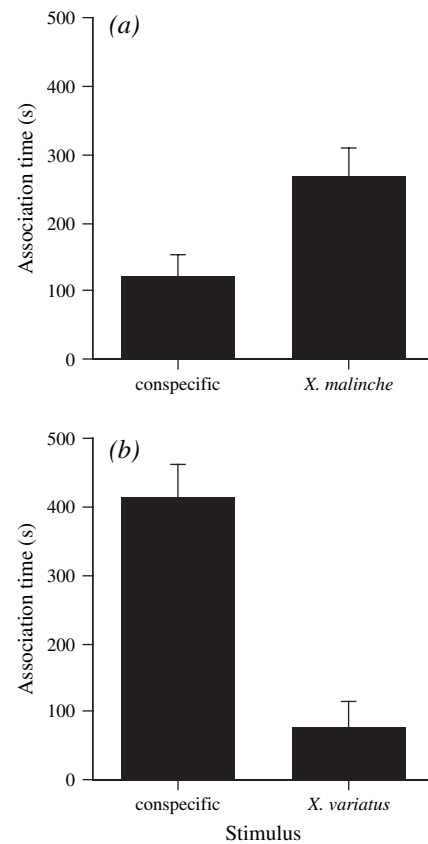


Figure 2 Association time (mean \pm SE) of male *Xiphophorus birchmanni* with female odor cues of (a) conspecific versus *Xiphophorus malinche* and (b) conspecific versus *Xiphophorus variatus*.

Given our results, we considered the possibility that the female *X. variatus* used to create the stimulus water may have been unresponsive and/or were not releasing any cues. To address this possibility we subsequently offered male *X. variatus* (mean SL \pm SD = 41.2 \pm 2.15 mm) the choice between the *X. variatus* stimulus and water and found that male *X. variatus* were significantly attracted to the stimulus of their own species (time with *X. variatus* = 334.2 \pm 40.5 s, water = 169.6 \pm 39.8 s; Wilcoxon signed-rank test: $z = 1.99$, $n = 10$, $p = .047$). We, therefore, ruled out the possibility that the lack of response of male *X. birchmanni* to *X. variatus* had been due to a problem with females used to collect the *X. variatus* stimulus.

Visual preference tests

Male *X. birchmanni* did not discriminate between size-matched, sexually mature female *X. birchmanni* and *X. malinche* based on visual cues (mean association time \pm SE with female *X. birchmanni* = 257 \pm 33 s, *X. malinche* = 221 \pm 29 s; Wilcoxon signed-rank test: $z = 0.31$, $n = 17$, $p = .76$).

DISCUSSION

Males have generally been regarded as fairly indiscriminate in their mating preferences. The male *X. birchmanni* in our study, however, not only discriminated among conspecific and heterospecific odor stimuli but also showed a complex array of preferences that has previously been documented only in females. First, male *X. birchmanni* differed in their response to the odor cues of different species. Males were attracted to odors from female conspecifics and the closely related,

allopatric *X. malinche*. They did not, however, associate more with the odor cues of the sympatric platyfish, *X. variatus*, over plain water, suggesting that male *X. birchmanni* do not recognize *X. variatus* as potential mates. Second, male preference for conspecifics also varied depending on which heterospecific was offered as an alternative. When given the choice between the conspecific stimulus and that of the platyfish, male *X. birchmanni* strongly preferred the conspecific odor. However, when offered odor cues of *X. malinche*, male *X. birchmanni* actually showed a weak preference for the heterospecific.

Although female preference for heterospecifics is not uncommon, very few studies (if any) have reported mate preference for the cues of another species in males. More typically, studies examining male mate recognition tend to find either a preference for conspecifics or no preference. In *Pieris* butterflies, for example, males of one species, *Pieris occidentalis*, discriminated against heterospecific females in favor of conspecifics (Wiernasz, 1995). In contrast, males in another species, *Pieris protodice*, showed no interspecific mate choice. A similar result was reported for male response to female sex pheromones in two species of gourami, *Trichogaster trichopterus* and *Trichogaster pectoralis* (McKinnon and Liley, 1987). It is unclear why male *X. birchmanni* may be more attracted to *X. malinche*. Work on female mate recognition in other species suggests that individuals could be drawn to novel traits that are not expressed in their own species (e.g., Basolo, 1995; Ryan and Rand, 1990) or, if expressed, are present in a more intense form in heterospecifics (Pfennig, 2000).

The structure of pheromones in *Xiphophorus* is unknown. Females in another poeciliid fish, the guppy, *Poecilia reticulata*, release an ovarian pheromone after parturition (Crow and Liley, 1979). Sex hormones—steroids and prostaglandins—have been implicated as important chemical cues in several fish species (Stacey et al., 2003). Perioovulatory females may sequentially release a preovulatory steroid pheromone and/or a postovulatory prostaglandin pheromone that can dramatically affect male behavior and physiology. In goldfish, Poling et al. (2001) found that the three steroid components of the female preovulatory pheromone each had distinct effects on male behavior. In several poeciliid species, females advertise their fertility through odor cues (Park and Propper, 2002; Sumner et al., 1994). Males, in turn, may be selected to discriminate among females based on these cues. The weak preference of *X. birchmanni* males for *X. malinche* odors, however, suggests that a species-typical component of the *X. birchmanni* odor cue is not necessary to provoke a male sexual response. In order to explain this rather counterintuitive result, we need to characterize the olfactory cue in these fishes.

Regardless of which cues may be involved, a preference for the heterospecific stimulus has important consequences. The result of our visual preference trials suggests that male *X. birchmanni* do not discriminate between *X. birchmanni* and *X. malinche* based on visual cues. If they rely, instead, on chemical cues, it is possible that their preferences could mediate hybridization with *X. malinche*. This would, of course, depend on whether female *X. malinche* are permissive. Although female mate recognition in *X. malinche* has not yet been tested, it is interesting to note that both species often hybridize in nature when they occur in sympatry (Rosenthal et al., 2003).

The degree of any geographic overlap between species may play an important role in the evolution of male preference for conspecific females. Specifically, selection may favor divergence of mate recognition systems in areas of sympatry between congeners if there is a high enough fitness cost to individuals who mate with heterospecifics (Pfennig, 2000). This, in turn, may cause individuals to discriminate more strongly in favor of conspecifics (or against the heterospecific) when offered a closely related but sympatric heterospecific as

a prospective suitor. For example, male sailfin mollies, *Poecilia latipinna*, discriminated less strongly against females of the gynogenetic *Poecilia formosa* as the geographic distance from sympatric populations increased (Gabor and Ryan, 2001). In our study, we used a population of *X. birchmanni* that was allopatric with *X. malinche* but sympatric with *X. variatus*. A preference for *X. birchmanni* may therefore be unsurprising when the alternative is the odor cues of sympatric *X. variatus* (Gabor and Ryan, 2001; McKinnon and Liley, 1987). Future studies could, perhaps, explore male responses using females from populations of *X. malinche* and *X. variatus* that are both sympatric and allopatric with *X. birchmanni*.

Phylogenetic relationships may also play an important role in the evolution of species recognition. In a study examining the response of *Streptopelia* doves to interspecific vocalizations, de Kort and ten Cate (2001) found that females were more responsive to the calls of closely related heterospecifics. Similarly, in swordtails, McLennan and Ryan (1999) showed that females of two closely related species, *Xiphophorus cortezi* and *X. nigrensis*, were more strongly attracted to the olfactory cues of each other than they were to the cue of a more distantly related species, *Xiphophorus montezumae*. Our results suggest a similar pattern of species recognition in males. There are, at present, several alternative hypotheses concerning the phylogenetic relationship of swordtail fishes. In the context of our study, however, one thing is uncontroversial: all phylogenetic hypotheses agree that *X. birchmanni* and *X. malinche* are more closely related to one another than either are to the platyfish, *X. variatus* (reviewed in Morris et al., 2001). Notwithstanding the possible effects of geographic overlap, phylogenetic distance could thus provide another plausible explanation for why *X. birchmanni* shows a strong preference for conspecifics when offered *X. variatus*. It cannot, however, explain why *X. birchmanni* males show a preference for the scent of a heterospecific. All other things being equal, we might expect males to have the greatest difficulty when distinguishing between conspecific odor and the scent of females from their sister species (i.e., show “no choice”). A preference for the heterospecific is, however, unexpected.

Male mate choice, though little studied, is no less important than female choice. Although females are typically regarded as the choosier sex, we now know that males can also be choosy (Gowaty et al., 2003). This relatively recent revelation can have important evolutionary implications. Within a given system, it is possible for members of one or both sexes to be attracted to conspecifics, show no species preference, or even prefer heterospecifics (Wong et al., 2004). The match or mismatch between male and female preference patterns could have important evolutionary consequences. Lande et al. (2001) argued, for example, that mutual male and female mate choice could lead to rapid sympatric speciation by sexual selection. Sexual asymmetry in mate choice, meanwhile, can lead to sex-biased asymmetry in hybridization with heterospecifics (Wirtz, 1999). Finally, preference asymmetries between the sexes can yield opposing effects of sexual selection on traits that are genetically correlated between the sexes (Wiernasz, 1995). Theoretical and empirical studies of mate choice should thus consider the role of both male and female preferences in structuring evolutionary outcomes.

We thank F.J. García de León, R. Struminger, J. Tovar Ortiz, and the Lara family for assistance in the field and D.G. Chapple, S. Downes, D. McLennan, L. Rowe, J. Stapley, M. Zuk, and anonymous reviewers for comments on the manuscript. We also thank the Government of Mexico for permission to collect fish. Finally, we thank C. O’Keefe, Z. Khalifa, and C. Schacter for assistance in the laboratory. B.B.M.W. is supported by a Sir Keith Murdoch Fellowship and H.S.F. by a Palmer McLeod Fellowship.

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