Conspecific mate recognition in swordtails, *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues

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Abstract. The swordtails *Xiphophorus nigrensis* and *X. pygmaeus* are allopatric sister species. Females of both species prefer *X. nigrensis* males because these males court while *X. pygmaeus* males do not. When exposed only to male odours, this mating asymmetry is not maintained; females of both species prefer conspecific mates. When female *X. pygmaeus* are exposed to both visual and olfactory cues of conspecific and heterospecific males, neither the mating asymmetry nor the conspecific preference is maintained; females fail to exhibit a preference. *Xiphophorus nigrensis* females do not distinguish between odours of large and small conspecifics, as they do in experiments restricted to visual cues. Thus, species mate recognition is influenced differentially by the independent divergence of cues used in different sensory modalities.

Females usually recognize and prefer to mate with conspecifics rather than heterospecifics. Mate recognition can involve information from different sensory modalities. The coordinated divergence of such cues is an integral part of the speciation process, and thus has always been of considerable interest to evolutionary biologists (Dobzhansky 1937; Mayr 1963; West-Eberhard 1983).

Contrary to suggestions that courtship functions to preserve species integrity (e.g. Dobzhansky 1937), some species-pairs exhibit asymmetries in mate preference. Although most common among flies in the genus *Drosophila* (reviewed in Ehrman & Wasserman 1987), this phenomenon occurs in other taxa, including fish. For example, female sticklebacks, *Gasterosteus* spp., preferred males with red versus black nuptial coloration, even if these males were heterospecific (McPahil 1969; Moodie 1972). Similar asymmetric preferences occur among two sister species of swordtails, *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae; Ryan & Wagner 1987). Females of both species preferred male *X. nigrensis*. This preference was due to the presence of complex courtship behaviour in most *X. nigrensis* and the absence of such behaviour in *X. pygmaeus*. Thus although mating behaviour has diverged between the species, there is a mating asymmetry that results from lack of divergence in female preference. Females of both species prefer courting males, and this preference is thought to be a shared ancestral trait (Ryan & Wagner 1987). This preference also leads to mate discrimination

within a species. Not all male *X. nigrensis* court. Males can be in one of three size classes, and males in the smallest size class, like the *X. pygmaeus* males, do not exhibit courtship (Ryan & Causey 1989); female *X. nigrensis* preferred larger, courting conspecific males to smaller, non-courting conspecifics (Ryan, in press a; Ryan et al., in press).

Mate recognition need not be restricted to one sensory modality. For example, odours can be important in species recognition in many animals (e.g. insects, Schneider 1969; mammals, Eisenberg & Kleiman 1972; fish, Chien 1973; Stacey et al. 1986). To influence mate recognition, odours need not be sexual pheromones. Instead they could provide cues for assessing individuals as being different or similar to one's self (e.g. Waldman 1985). Odours can also provide the basis for asymmetric mating preferences. Males of one species of gourami, *Trichogaster*, preferred the female conspecific odour, while the males of another species were equally responsive to the odours of both species (McKinnon & Liley 1987).

The purpose of our study was to investigate the use and interaction of two sensory modalities in mate recognition. Because female *X. pygmaeus* prefer courting heterospecifics to their own non-courting males, the divergence of signals and preferences in the visual modality has not been sufficient for ensuring conspecific preferences. We investigated how mate preferences are influenced by olfactory cues alone, and when in combination with visual cues by addressing the following questions.
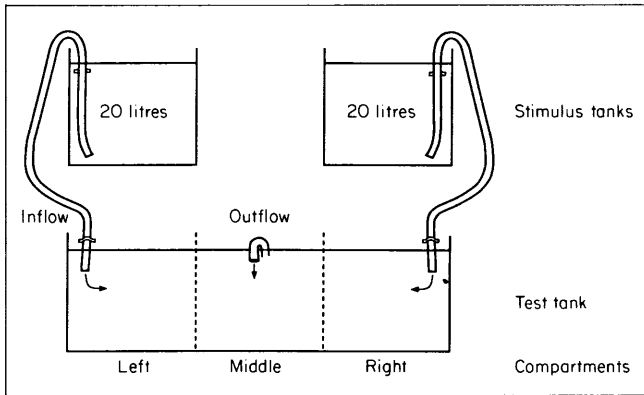
Figure 1. A diagram of the testing apparatus.

First, has divergence of cues and preferences in the olfactory modality been sufficient for conspecific preferences? Second, how do these cues, when combined with visual cues, influence the mating asymmetry? Third, do these cues also result in intraspecific mate preferences? These results should provide insights into the evolutionary divergence of cues decoded in different sensory modalities that are used in species recognition, and thus into the process of speciation.

METHODS

Study Animals

All animals tested were either field captured or progeny of field-captured animals. *Xiphophorus nigrensis* were collected from the Rio Choy, near Tamuin, San Luis Potosi, Mexico. *Xiphophorus pygmaeus* were collected from the Rio Axtla near Chimalaco, San Luis Potosi, Mexico. The standard length (SL, snout to hypural plate) of all fish used in the experiments was measured with dial calipers.

Experimental Apparatus: Odour Only

The experimental apparatus consisted of two 20-litre aquaria (stimulus tanks) containing the males that provided the olfactory stimuli (stimulus fish) and one 120-litre tank (test tank) containing the female to be tested for olfactory discrimination (test fish; Fig. 1). The stimulus tanks were situated behind and above the test tank. Each had a plastic hose measuring 2·5 mm in internal diameter fixed to the tank such that water from the stimulus tanks

could flow into opposite back corners of the test tank. A hose measuring 8·5 mm in internal diameter located in the middle of the test tank prevented overflow (Fig. 1). The test tank was divided into three equal compartments measuring $30 \times 30 \times 20$ cm each by lines drawn on the outside of the tank. The two end compartments (stimulus compartments) contained an inflow tube from one of the stimulus tanks. The bottoms of these inflow tubes were placed about 10 cm below the water's surface and faced away from the centre of the tank.

The water temperature was $20 \pm 1·5°C$ and we used a 12:12 h light:dark cycle. Water in the test and stimulus tanks was aerated and charcoal filtered for at least 18 h prior to use. Water was not aerated or filtered during the experiments.

Testing Procedure: Odour Only

The male stimulus fish were placed in their respective stimulus tanks 2 days prior to the experiment. The female test fish were isolated individually in single plastic containers 2 days before their first test; they remained isolated until the end of all experiments, approximately 1 month later. The plastic containers were transparent, thus the isolated test fish could see one another. To avoid contamination by food odours, none of the fish was fed in any of the experimental tanks. When not being used in experiments, the stimulus fish were fed in separate tanks and two females were added to these feeding tanks to maintain the sexual motivation of the males. The same stimulus fish were used for all experiments.

The rate of flow from each stimulus tank was 8·5 ml/s. Observation of water dyed with cresyl

violet acetate showed that flow within the test tank was symmetric. To avoid contamination by odours between tests, the test tank was emptied, rinsed and refilled with uncontaminated water between experiments. The stimulus tanks were thoroughly washed and dried between tests while the stimulus fish were housed in holding tanks. The water flows from the stimulus tanks were switched between the ends of the experimental tank to control for side biases.

Each test fish was placed in the test tank 60 ± 5 min prior to the experiment. At the beginning of the experiment, the water started flowing simultaneously from each stimulus tank into the test tank. As the water level increased in the test tank, water emptied through the outflow. Recording began when the test fish moved at least one body length. Recording then continued for 10 min. The same observer conducted all experiments, and the following were recorded: (1) number of lunges, defined as when the fish swam in a short, darting motion toward the inflow tubes; and (2) time associated with each stimulus, which was the amount of time the fish spent in either of the stimulus compartments.

Experiments were conducted every other day in the morning and afternoon. Females were tested twice for each stimulus pair with the stimuli on different sides of the tank to control for side biases. Each test of the same female was on a different day.

Experimental Apparatus: Odour and Vision

Preference of *X. pygmaeus* females for *X. pygmaeus* or *X. nigrensis* males was determined in experiments in which females had access to both olfactory cues and visual cues. The testing apparatus combined aspects of the olfactory experiments, described above, and the experiments in which females were limited to visual cues, as in Ryan & Wagner (1987). The 1600-litre test aquarium was divided into five equal sections measuring $45 \times 90 \times 41$ cm by lines drawn on the outside of the tank. In addition, inside the tank the sections at each end were separated by Plexiglas from the three central sections; the end compartment contained the stimulus males. Two stimulus tanks containing either *X. pygmaeus* or *X. nigrensis* males were used to deliver odours into the test tank; the procedures followed were as described above. The water from the stimulus tanks was delivered to the inside of the

Plexiglas that partitioned the stimulus compartment from the three central compartments; thus, the water from the stimulus tanks reached the central compartments of the tank which contained the female.

Testing Procedure: Odour and Vision

Four males of either *X. pygmaeus* or *X. nigrensis* were maintained in each stimulus tank as described above. Ten minutes prior to testing, one male from each of the stimulus tanks was placed in each of the end sections. An opaque cylinder measuring 11 cm in diameter and higher than the water column was placed in the middle section. A female was placed in the cylinder and allowed to acclimate for 10 min.

The experiment was begun by starting the flow of water from the stimulus tanks into the test tank, and lifting the opaque cylinder to allow movement of the female. The water from the stimulus tank containing *X. pygmaeus* males was delivered to the end of the test tank that contained the *X. pygmaeus* male in the stimulus compartment. Likewise, the water from the stimulus tank containing *X. nigrensis* males was delivered to the end of the test tank that contained the *X. nigrensis* male. In this manner, females were exposed to visual and olfactory cues of the same species simultaneously at the same end of the tank. The amount of time that the female spent in each of the two sections that were adjacent to each male was recorded for 10 min. The females' average response over two tests, each on a different day and between which the stimuli were alternated, was used for statistical analysis, thus controlling for side biases.

Statistics

The null hypothesis of no difference in the response was evaluated with a paired *t*-test. Differences in the strength of response between experiments and between species were evaluated by a two-way ANOVA. A significant interaction term would reject the null hypothesis of no difference in the strength of the response.

RESULTS

Experiment 1: Female *X. nigrensis*: Conspecific Odours versus Control

Eight female *X. nigrensis* were tested for their response to the stimulus water from large male
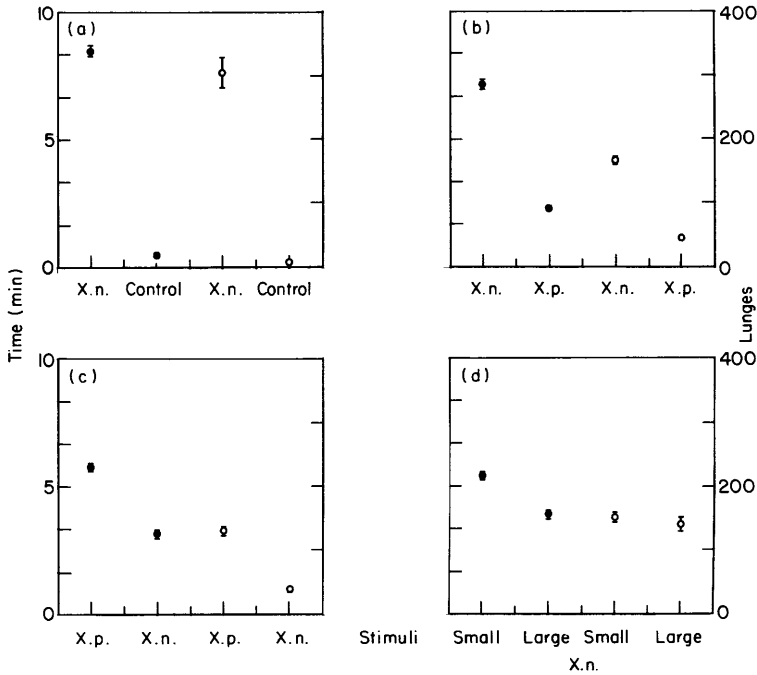
Figure 2. The average amount of time ($\pm$ SE) spent in each stimulus compartment ($\bullet$) and the average number of lunges to each inflow valve ($\bigcirc$) for each of two stimuli presented to female *X. nigrensis* (a, b, d) and *X. pygmaeus* (c). All stimuli are males of the species indicated with the exception of Control, which is uncontaminated water. X.n.: *X. nigrensis*; X.p: *X. pygmaeus*.

X. nigrensis (> 31 mm SL) versus water uncontaminated by fish odour. In these tests, the stimulus males were removed immediately prior to the experiments to control for the possibility of any acoustic cues. These experiments were conducted blind and stimuli were assigned randomly among experiments; the observer did not know which stimulus tank contained the males. The purpose of this experiment was to control for effects of water current and possible acoustic cues that might confound preference for odour cues, and also to compare with the results from experiment 2.

Females spent significantly more time in the compartment associated with the male odour than in the compartment associated with the uncontaminated water ($t = 9.23$, $P = 0.000$; Fig. 2a). Females also exhibited more lunges to the inflow tube with male odours than to the control inflow tube ($t = 4.50$, $P = 0.003$; Fig. 2a).

Experiment 2: Female *X. nigrensis*: Conspecific versus Heterospecific Odours

Seventeen females were tested for their response to the odours of the same large conspecific males used in experiment 1 versus the odour of five male *X. pygmaeus* (< 28 mm SL). Females showed a strong preference for the odour of conspecific males. This was true whether preferences were estimated as the time spent in association with each stimulus ($t = 4.03$, $P = 0.001$) or the number of lunges toward each stimulus ($t = 3.99$, $P = 0.001$; Fig. 2b).

We compared the responses of *X. nigrensis* in experiments 1 and 2 to determine if the strength of the response toward the conspecific stimulus was influenced by the alternative stimulus. There was a significant interaction between the response and the experiment in both the amount of time ($F = 4.46$, $df = 1,46$; $P = 0.040$) and the number of lunges ($F = 7.84$, $df = 1,46$; $P = 0.007$).

Experiment 3: Female *X. pygmaeus*: Conspecific versus Heterospecific Odours

Thirteen female *X. pygmaeus* were tested for their response to odours of conspecific and heterospecific males, using the same males as in experiment 2. The *X. pygmaeus* females exhibited significantly more lunges toward the inflow tube with the conspecific

odour ($t = 2·75$, $P = 0·018$; Fig. 2c). The average amount of time spent in the conspecific stimulus compartment was greater than that spent in the heterospecific compartment, but this difference was not statistically significant ($t = 1·98$, $P = 0·071$; Fig. 2c).

We also compared the responses of *X. nigrensis* females (experiment 2) with the responses of *X. pygmaeus* females (experiment 3) to ascertain if the conspecific response was significantly different between the species. The two species did not exhibit significant differences in the degree to which they were biased between the conspecific and hetero-specific odours either in the amount of time spent in stimulus compartments ($F = 2·44$, $df = 1,56$, $P = 0·124$) or in the number of lunges toward each in-flow tube ($F = 0·47$, $df = 1,56$, $P = 0·490$; Fig. 2b, c).

Experiment 4: Female *X. pygmaeus*: Conspecific versus Heterospecific Odours plus Visual Cues

Sixteen *X. pygmaeus* females were presented with both the visual and olfactory cues of *X. pygmaeus* and large *X. nigrensis* males (> 31 mm SL). There was no statistically significant difference in the amount of time females spent with each species (*X. nigrensis*: $\overline{X} \pm \text{SE} = 4·6 \pm 0·63$ min; *X. pygmaeus*: $\overline{X} \pm \text{SE} = 3·12 \pm 0·50$ min; $t = 1·56$, $P = 0·139$).

Experiment 5: Female *X. nigrensis*: Conspecific Large Versus Small Male Odours

The same seventeen female *X. nigrensis* used in experiment 2 were presented with odours of the five large *X. nigrensis* males (> 31 mm SL) used in that experiment versus the odour of five small *X. nigrensis* males (< 26 mm SL). There were no significant differences in either time spent in each stimulus compartment ($t = 1·11$, $P = 0·283$) or in the number of lunges toward each inflow tube ($t = 0·18$, $P = 0·859$; Fig. 2d).

DISCUSSION

Female *X. nigrensis* respond to odours of con-specific males in the absence of other cues. Thus, as with many other fish (Chien 1973; Crapon de Caprona 1980, 1982; Liley & Stacey 1983; Stacey et al. 1986), *X. nigrensis* both produces and perceives odours. Male odours also differ among species, and both female *X. nigrensis* and *X. pygmaeus* perceive such differences. The response of *X. nigrensis* toward the conspecific odour was stronger if the alternative stimulus was water rather than the odour of *X. pygmaeus* males. This suggets that although female *X. nigrensis* prefer the odour of their own males, they do perceive the heterospecific odour.

This study demonstrates that male odours and the preference for these odours have diverged between these two sister species. We do not suggest that these odours are sexual pheromones, only that females use these cues in recognition. The diver-gence of this species pair was probably due to a vicariant event, and these species probably have remained allopatric since (Rosen 1978, 1979). Thus, divergence in both olfactory cues and the perception of such cues sufficient to allow specific mate recog-nition seems to have occurred in allopatry without the need for reinforcement or reproductive charac-ter displacement to enhance such differences, as suggested by Dobzhansky (1937) in general, and for cichlids, specifically (Mayr 1984; see also Crapon de Caprona & Fritzsch 1984). This is consistent with the notion that the evolution of specific mate recognition systems can occur in allopatry (e.g. Paterson 1985; Ryan, in press b).

Female preference for male odours was sym-metric for both *X. nigrensis* and *X. pygmaeus* in this study, and differs from preference based on visual displays, which is asymmetric. *Xiphophorus nigrensis* and *X. pygmaeus* males have diverged in courtship display, due either to a loss of behaviour in *X. pygmaeus* or a gain in *X. nigrensis* (Ryan & Causey 1989), while female preference has not diverged (Ryan & Wagner 1987; Ryan, in press a). Ryan & Wagner (1987) suggested that the similar female preference of these two species is due to a shared ancestral trait rather than evolutionary convergence. Although there has been divergence in mating behaviour between the two species, the mating asymmetry results from the lack of divergence in female preferences.

In nature, females are exposed to both olfactory cues and visual cues when assessing mates. Our ex-periments show that when both cues are available, female *X. pygmaeus* did not exhibit a preference, thus neither the mating asymmetry nor conspecific preference was maintained. However, on average, females spent slightly more time with the hetero-specifics. The data do not allow us to accept the null hypothesis, i.e. that there is no preference, but we

do suggest that the preference of *X. pygmaeus* females for *X. nigrensis* males based on visual cues (Ryan & Wagner 1987) is at least made less apparent by the presence of odours, since Ryan & Wagner (1987) demonstrated a statistically strong heterospecific preference in a study with smaller sample sizes (e.g. $N = 10$, $t = 10.9$, $P = 0.001$).

Different cues and the associated sensory modalities used in mate recognition may not all function at the same level of discrimination; some may be restricted to intraspecific discrimination, while others might be more general, such as localization of conspecifics. It might be that olfactory cues, which can be carried long distances by water currents (e.g. salmon, *Salmo salar*, Nordeng 1971), will guide females to areas containing conspecific males, and further female assessment of mates might be based on courtship. Thus, the divergence of different recognition signals, either in allopatry or sympatry, can have quite different consequences for species recognition and sexual selection (Ryan, in press b). These studies of mate preference in the *X. nigrensis–X. pygmaeus* species pair (Ryan & Wagner 1987; Ryan & Causey 1989; Ryan, in press a; present study) also show that species recognition cues decoded in different sensory modalities do not evolve in a coordinated fashion, but instead can change in a haphazard manner without reference to other cues. This suggests that the potential for reproductive isolation through ethological differences may in part depend on the underlying sensory mechanisms used in mate recognition (see also Ryan 1986).

ACKNOWLEDGMENTS

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