INTERSPECIFIC RECOGNITION AND DISCRIMINATION BASED UPON OLFACTORY CUES IN NORTHERN SWORDTAILS

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Abstract.—Female *Xiphophorus montezumae* were attracted to olfactory cues from conspecific and heterospecific (*X. cortezi* and *X. nigrensis*) males when given a choice between the stimulus and water. When given a choice between conspecific and heterospecific cues, females only demonstrated a strong preference for the conspecific stimulus when it was matched against *X. nigrensis*. Female *X. nigrensis* were attracted to olfactory cues from their close relative, *X. cortezi*, but did not respond to cues from the more distantly related *X. montezumae*. They preferred the scent of their own males to the olfactory cues of both heterospecific species. Our results indicate that *X. cortezi* and *X. nigrensis* share an apomorphic change in some aspect of their olfactory cue-receiver system that is not shared with *X. montezumae*. We also uncovered an asymmetry in response based on olfactory stimuli in these fishes: *X. montezumae* is moderately attracted to the cue from *X. nigrensis*, whereas *X. nigrensis* does not respond to the cue from *X. montezumae* at all.

Key words.—Mate choice, olfactory cues, phylogeny, swordtail fishes, *Xiphophorus*.

The way in which females choose their mates has fascinated researchers for over a century. That fascination has tended to follow two separate lines of research involving the role of female choice in the evolution of either species recognition and isolation (Mayr 1963; reviewed in Ryan 1990) or exaggerated male nuptial traits via sexual selection (Darwin 1859, 1871; see examples in Andersson 1994). Recently, however, a number of authors have suggested that the dichotomy between sexual selection and species recognition is unjustified, that both are part of a continuum of decisions made by females as they move through the environment searching for an appropriate mate (Verrell 1988; Ryan 1990; Zahavi 1991; Cronin 1992; Ryan and Rand 1993; Endler and Houde 1995).

Mate choice is usually studied on a macroevolutionary level by asking whether a female is capable of discriminating between conspecific and heterospecific males. There are three possible outcomes to such trials. First, the conspecific and heterospecific cues may be so similar that the female cannot tell them apart. Presentation of these two cues offers the female a choice between equivalents, so she does not discriminate (no preference). Second, the female may be strongly attracted to a cue that is only produced, or produced in a more intense version, by heterospecific males. When given a choice between the conspecific and heterospecific based upon that cue alone, she will discriminate in favor of the heterospecific (Ryan 1990; Ryan and Rand 1990; Ryan and Keddy-Hector 1992 and references therein). In both of the preceding cases, females within a clade retain the plesiomorphic (ancestral) mating response and thus run the risk of making mistakes when confronted with appropriate heterospecific males (Cracraft 1983). In zones of contact between such species, we would expect to find either extensive introgression or the divergence of one or both of the sympatric populations if there is a substantial cost to interspecific hybridization (Dobzhansky 1940; Ryan and Wagner 1987; Hos-

tert 1997). The maintenance of a plesiomorphic female mating preference is thus expected to occur more often in allopatry, regardless of whether the male cue is itself plesiomorphic or apomorphic (derived) within the clade (e.g., Baird et al. 1992).

The third outcome, and the one that we expect to see most often, is female discrimination in favor of the conspecific male. Such species-specific mate choice, however, could itself evolve in two very different ways depending upon the female's response to the heterospecific cue on its own. First, the signal and receiver may be either genetically correlated or genetically coupled (for reviews see Butlin and Ritchie 1989; Boake 1991) and diverging with each speciation event. From the female's perspective, there is no choice in a conspecific versus heterospecific trial because she does not recognize the heterospecific cue. Second, females may be attracted to the cue produced by a heterospecific, but demonstrate a preference for their own males when given a choice between the two. In this situation, the female perceives the heterospecific cue as being meaningful and only rejects it in the presence of other (conspecific) information (Backwell and Jennions 1993; Ryan and Rand 1993, 1995; see discussion in Gerhardt et al. 1994). Given the initial attraction, there is a potential for females to make mating mistakes in this kind of system if they ever come into contact with the appropriate heterospecific. The potential to make mistakes, in turn, has important ramifications for studies of speciation and the evolution of mate recognition systems.

Although there are a plethora of studies documenting various species' ability to discriminate between a conspecific and a heterospecific cue, most researchers have not asked if their test species would react to the heterospecific cue on its own (but see studies of advertisement calling in bush crickets: Gwynne and Morris 1986; hylids: Backwell and Jennions 1993; Gerhardt et al. 1994 and references therein; and Túngara frogs: reviewed in Ryan and Rand 1995). Of that handful, only one study has investigated the evolution of interspecific mate recognition within a phylogenetic framework. Ryan and Rand (1995 and references therein) examined female Túngara frogs' responses to the male advertisement call

from extant species and reconstructed ancestral nodes within the *Physalaemus pustulosus* clade. They discovered that the strength of preference for the conspecific increased with increasing phylogenetic distance between the female and the heterospecific male. Attraction to the heterospecific cue on its own, however, was better predicted by overall call similarity and was not correlated with phylogeny. Apparently female Túngara frogs are attracted to a component of the male call that has changed very little across the clade (i.e., is plesiomorphic or ancestral), but discriminate based on a call component that has evolved more rapidly (i.e., is apomorphic or derived).

To investigate this phenomenon further, we conducted a comparative study of the response by females to olfactory cues produced by males from three species within the northern swordtail clade (*Xiphophorus montezumae*, *X. cortezi*, and *X. nigrensis*). The results of our first study indicated that female *X. cortezi* were attracted to male-based olfactory cues from all three species, but discriminated in favor of the conspecific stimulus when given a choice between the conspecific and heterospecific (McLennan and Ryan 1997). In this paper, we examine *X. montezumae* and *X. nigrensis* females' response to olfactory cues produced by conspecific and heterospecific males to determine whether they show the same pattern of response as *X. cortezi* females (attraction to the heterospecific, preference for the conspecific) and whether there is a relationship between the degree of relatedness to the heterospecific and the strength of either attraction to the heterospecific or preference for the conspecific cue.

MATERIALS AND METHODS

Phylogenetic Relationships

The genealogical relationships within the northern swordtail clade remain ambiguous. However, the competing phylogenetic hypotheses do agree on one point critical to our study: *X. cortezi* and *X. nigrensis* are more closely related to one another than either is to *X. montezumae*. The morphological plus electrophoretic-based tree (Rauchenberger et al. 1990) places *X. cortezi* and *X. nigrensis* five and six nodes from *X. montezumae*, respectively, whereas the molecular-based tree (Meyer et al. 1994) places *X. cortezi* and *X. nigrensis* equidistant (four nodes) from *X. montezumae*. We will use both trees in this study to disentangle explanations that do not vary from explanations that do vary according to tree topology.

Study Animals

Xiphophorus montezumae and *X. cortezi* individuals were collected from Río Gallinas, Agua Buena, Mexico and Río Huichihuayán, Huichihuayán, Mexico, respectively, in April, 1995. *Xiphophorus nigrensis* subjects were progeny of field-captured animals, collected from Río Choy, Mexico and raised in outdoor ponds at Brackenridge Field Lab, Austin, Texas. Fish were maintained in 227-L stock tanks in the laboratory from May to July, 1995. Lights were set on a 12:12 L:D cycle. Temperature fluctuated between 20° C and 22°C in the stock tanks. Fish were fed ad libitum on live brine shrimp nauplii and Tetra Min flakes twice daily. Standard length (tip of snout to base of caudal peduncle) was measured with dial calipers.

Individual test females were housed in 4.5-L jars covered with coarse nylon mesh and placed in water-filled 22-L tanks (four jars to a tank). Filters in each tank ensured that the water circulating throughout and around the jars was aerated, cleaned, and temperature controlled. The females could see each other, but were isolated from other fish. They were fed ad libitum on brine shrimp nauplii at 0700 h and 1700 h daily. The jars were emptied and refilled every day to remove excess food.

Producing the Stimulus

We placed 10 sworded, conspecific males, representing much of the male size range in the population (*X. montezumae*: 39.7–57.4 mm; *X. cortezi*: 25.9–43.9 mm; *X. nigrensis*: 28.8–38.3 mm), into a 45-L stimulus tank filled with 32 L of water and covered it with glass to prevent contamination from air-borne odors. The water had been aerated and charcoal-filtered for 24 h prior to adding the males. A larger holding tank containing female conspecifics, was placed beside the stimulus tank to arouse the males. Males remained without food in the stimulus tank for 24 h and then were moved to the holding tank. The stimulus water was used as needed for a maximum of 48 h before being discarded. Preliminary tests revealed no decline in female response to the stimulus over that period. We used the same group of males for each species to produce stimulus as required. Control water was produced following the pattern for stimulus water: 32 L of water was aerated and charcoal-filtered in a glass covered tank for 24 h, allowed to sit undisturbed for another 24 h, used for 48 h, and then discarded.

Experimental Set-up

The experimental apparatus consisted of two 70-L tanks (test tanks; 100 × 26 × 30 cm) and four 4.5-L jars (stimulus jars). The stimulus jars were placed above and behind the test tanks (Fig. 1). A stimulus delivery system (SDS) was constructed by attaching a 12-mm glass pipette to a piece of 2.5-mm silicone tubing. The SDS was secured by slipping the open end of the silicone tubing through a piece of 6.0-mm tubing attached to the bottom of the stimulus jar and slipping the pipette through a piece of 6.0-mm tubing attached to the side of the test tank. Each SDS was used for only one stimulus type. The tip of the pipette sat 0.5 cm above the water surface. Fish, which were fed in stock tanks by injecting a large pipette full of brine shrimp into the tank, had learned to respond quickly to any surface disturbance. Dripping the stimulus above the water mimicked that disturbance and prompted the female to respond quickly and to move between the two sides. Provoking this response is critical in an olfactory experiment because the female must physically move throughout the entire tank to guarantee exposure to both stimuli.

Stimulus flow was initiated each day by injecting water from the test tank into the SDS, placing the open end of the tubing in the stimulus jar, and allowing the stimulus water to flow via gravity through the pipette into a drain for 60 sec. Flow rate, controlled by the use of metal screw clamps,
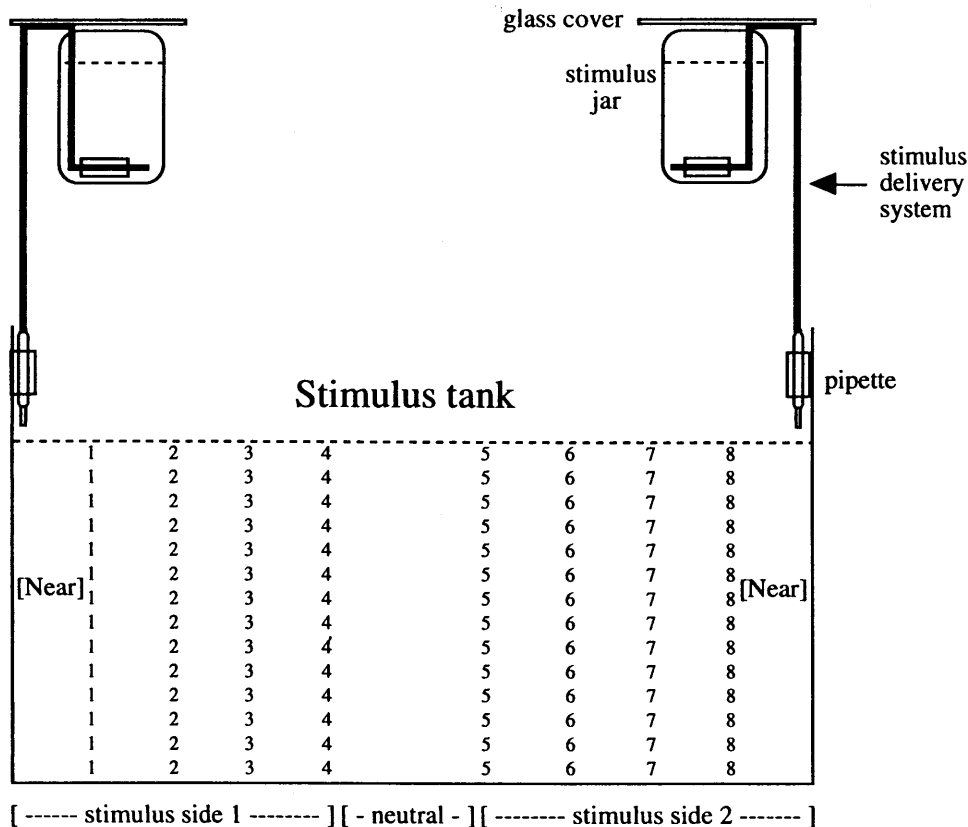
FIG. 1.   Experimental set-up (modified from McLennan and Ryan 1997).

was set at 1.2 ml/min. At the end of each trial, the pipette was placed back in the stimulus jar so the flow rate rarely needed to be adjusted throughout the test period. Observation of water dyed with food coloring showed that the stimulus flow within each tank was symmetrical, and that, in the absence of a test fish, the currents on either side of the tank had not met after 30 min. To control for side biases, the stimulus was assigned randomly to the left or the right side in each trial.

The test tank was marked on the outside with eight numbered, vertical lines 10 cm apart to pinpoint the female's position during video analysis. After each trial, the tank was scrubbed vigorously with a 3% solution of hydrogen peroxide and soap, rinsed with an intense jet of water concentrated along the silicone seams, and dried thoroughly. This appeared to remove all vestigial traces of the stimulus.

Experimental Procedure

A female was placed in the test tank, allowed to familiarize herself with her new surroundings for 30 min, then videotaped for 5 min (pretrial period). Stimulus flow was initiated by simultaneously slipping the pipettes into their respective holders on either side of the tank. Videotaping of the 5-min trial period began once the female had passed within 10 cm of each stimulus pipette. At the end of the trial, the female was returned to her holding jar and allowed one day of rest. The presentation of test stimuli followed the pattern: conspecific versus water (days 1–2); heterospecific 1 versus wa-

ter (days 3–4); heterospecific 2 versus water (days 5–6); conspecific versus heterospecific 1 (days 7–8); conspecific versus heterospecific 2 (days 9–10); and heterospecific 1 versus heterospecific 2 (days 11–12). To determine whether the female's behavior changed across the 12-day experimental period, pretrial tests were videotaped before the conspecific versus water trials (pretrial 1: first two days of the experiment) and before the heterospecific 1 versus heterospecific 2 trials (pretrial 2: last two days of the experiment).

Variables Scored

We analyzed the videotapes at a later date, and scored the following variables: (1) Total time: the time spent within 40 cm of each outflow pipette. The 20-cm portion in the middle of the tank was assigned a neutral status because it was often difficult to determine the females' orientation during the time she spent hovering there. (2) Percent of time near: the time spent within 10 cm of each outflow pipette/total time. (3) Percent of time interacting: the time spent engaged in diving plus nudging behavior divided by total time. Diving involved swimming rapidly up the side of the tank toward the stimulus outflow, turning sharply, and swimming rapidly toward the bottom. During nudging, the female made contact with the side of the test aquarium in a series of butts against the glass. A bout of nudging involved a series of rapid butts interspersed with prolonged swimming, with the nose in contact with the glass and all fins flared.

The nonparametric Wilcoxon signed-ranks matched pairs

test was used to evaluate the null hypothesis that there was no difference in the female's response to different cues. Given the relatively large number of tests being conducted, a sequential Bonferroni correction was applied to decrease the probability of committing a Type I error by chance alone (Holm 1979; Peres-Neto 1999). The correction ($\alpha \leq 0.05$) was applied to the pretrial, stimulus versus water, and choice between two stimuli trials. Data were analyzed using the software package Statview II version 4.5 (Abacus Concepts).

RESULTS

Pretrial

Females from both species displayed no bias in terms of the total time spent, the percentage of time spent near, or the percentage of time spent interacting on either side of the tank (Table 1). There was no difference between pretrial 1 and pretrial 2 in any of the preceding variables, indicating that the females did not adjust these aspects of their behavioral response across the 12-day experimental session.

Olfactory Stimulus versus Control Water

Xiphophorus montezumae females displayed a significant response to the olfactory stimulus from all species compared to control water for the three variables measured (Table 1). *Xiphophorus nigrensis* females responded to the olfactory stimulus from conspecific and *X. cortezi* males, but they did not distinguish between control water and stimulus water from *X. montezumae* males (Table 1).

We tested the null hypothesis that the strength of the female's response to the olfactory cue did not differ among the three stimulus species. An overall response value for two behavioural variables (time near and time interacting) y was calculated as: [(total time engaged in y on stimulus side − total time engaged in y on control side)/(total time engaged in y on stimulus side + total time engaged in y on control side)] × 100 (McLennan and Ryan 1997).

Xiphophorus montezumae females responded more strongly to olfactory cues from their own males than they did to cues from *X. nigrensis* (time interacting: $z = -2.33$, $P < 0.02$; time near see Fig. 2). There was no difference between their response to conspecific and *X. cortezi* males, nor did they differentiate between the two heterospecifics. *Xiphophorus nigrensis* females responded more strongly to olfactory cues from their own males than they did to cues from either *X. cortezi* (time interacting: $z = -3.01$, $P < 0.003$; Fig. 2) or *X. montezumae* males (time interacting: $z = -2.79$, $P < 0.005$; Fig. 2). Females also differentiated between the two heterospecifics, responding more strongly to *X. cortezi* than to *X. montezumae* (time near: $z = -2.10$, $P < 0.04$; time interacting: $z = -2.10$, $P < 0.04$).

Choice between Two Olfactory Stimuli

Female *X. nigrensis* demonstrated an unambiguous preference for the olfactory cue from their own males versus the cue from both heterospecific species for all three variables (Table 2). Female *X. montezumae*, on the other hand, displayed a preference for the scent of their own males versus *X. nigrensis*, but did not show a strong response to the cue

from their own males when *X. cortezi* was offered as the alternative (the significant results shown in Table 2 disappear after a Bonferroni correction). When given a choice between the two heterospecifics, female *X. nigrensis* spent more time overall on the *X. cortezi* side (Table 2).

We tested the null hypothesis that the strength of preference for the conspecific did not differ based on the heterospecific offered as an alternative. Preference strength was measured as the relative attraction to the conspecific versus the heterospecific cue for two behavioral variables (time near, time interacting) y and was calculated as: [(total time engaged in y on conspecific side − total time engaged in y on heterospecific side)/(total time engaged in y on conspecific side + total time engaged in y on heterospecific side)] × 100. In all cases Bonferroni corrections eliminated any significant differences. The general trend, however, was for females to display a stronger preference for the conspecific cue when it was matched against the least attractive of the two heterospecifics (Fig. 3).

DISCUSSION

When we study mate choice in the laboratory, we are really seeing the endpoint of a long process involving recognition (Is this a potential mate?) and discrimination (Which of these potential mates is the one for me?) (Ewing 1988; Ryan and Rand 1993). In a perfectly coevolving system, one in which recognition and discrimination evolve in nonoverlapping quantum leaps with each speciation event (interspecific gap: Backwell and Jennions 1993), we would expect every female in a clade to show a strong preference for conspecific males because heterospecific males would not be recognized as potential mates. Most, if not all, of the biological world, however, is one in which evolutionary change is influenced by the unique history of the evolving system; the past tends to leave its mark on the present (see references in Brooks and Wiley 1988). On a macroevolutionary time scale, one of the manifestations of the past in a mate choice context may be female recognition of heterospecific cues. When this happens, the interaction between interspecific recognition and discrimination may be very complex across the history of a group (e.g., Ryan and Rand 1995).

This is certainly true for the northern swordtail clade in which females from at least three species discriminate in favor of conspecific males based upon olfactory cues (Crapon de Caprona and Ryan 1990; McLennan and Ryan 1997; this study), even though they are attracted to the olfactory cues from heterospecifics (McLennan and Ryan 1997; this study). To provoke both recognition and discrimination in the receiver, signals must contain plesiomorphic (basis of interspecific recognition) and apomorphic (basis of interspecific discrimination) information. Research on other piscine species indicates that conjugated steroids from the seminal vesicle, testis, and urogenital tract and amino acids from the seminal vesicle, urine, and skin mucus of males act as powerful attractants to females (see discussion in McLennan and Ryan 1997). Resink et al. (1989a,b) suggested that species-specific responses in the African catfish, *Clarias gariepinus*, might be based on the diversification of either a conjugated gonadal steroid blend or a protein. It is thus possible that the

TABLE 1. Mean results (SD) of pretrial and mate recognition tests. All significant values were retained after a sequential Bonferroni correction.

| | *Xiphophorus montezumae* females ($n = 16$) | | |
	Total time (sec)	% time near	% time interacting
Pretrial tests			
(1) Left side	135.9 (63.2)	37.1 (21.4)	14.2 (16.7)
Right side	139.2 (71.4)	36.8 (19.7)	12.8 (19.1)
	ns ($P = 0.86$)	ns ($P = 0.84$)	ns ($P = 0.78$)
(2) Left side	146.7 (47.4)	38.2 (11.5)	19.4 (18.2)
Right side	148.7 (46.0)	39.0 (22.3)	17.0 (17.0)
	ns ($P = 0.71$)	ns ($P = 0.57$)	ns ($P = 0.60$)
Water versus conspecific	49.5 (28.9)	30.3 (19.1)	5.6 (11.1)
	250.5 (28.9)	71.1 (14.9)	34.1 (18.5)
	$z = -3.52, P < 0.0004$	$z = -3.46, P < 0.0005$	$z = -3.26, P < 0.002$
Water versus heterospecific 1[a]	63.9 (51.2)	33.0 (25.9)	4.6 (8.9)
	234.4 (51.1)	70.4 (16.8)	35.1 (24.0)
	$z = -3.52, P < 0.0004$	$z = -3.46, P < 0.0005$	$z = -3.26, P < 0.002$
Water versus heterospecific 2[b]	102.2 (36.5)	33.7 (18.6)	8.9 (9.2)
	197.8 (36.5)	55.7 (14.9)	31.1 (19.4)
	$z = -3.52, P < 0.0004$	$z = -2.53, P < 0.02$	$z = -2.95, P < 0.004$

[a] For both *X. montezumae* and *X. nigrensis* females, heterospecific 1 = *X. cortezi* males.
[b] For *X. montezumae* females, heterospecific 2 = *X. nigrensis* males; for *X. nigrensis* females, heterospecific 2 = *X. montezumae* males.

plesiomorphic, recognition component of the male swordtail olfactory cue is gonadal steroid-based, whereas the apomorphic, discrimination component is based on a particular blend of conjugated gonadal steroids or a protein. In other words, it is more probable that the olfactory "cue" is in fact a cue complex.

Most of the differences in female response to olfactory cues from conspecific and heterospecific males mirror the pattern of genealogical relatedness suggesting that *X. cortezi* and *X. nigrensis* are more closely related to each other than either is to *X. montezumae*. For example, *X. cortezi* (McLennan and Ryan 1997) and *X. nigrensis* were attracted to the olfactory cue from each other's males more strongly than they were to the cue from *X. montezumae*. These species discriminated more strongly in favor of the conspecific when offered *X. montezumae*, rather than each other's males as the
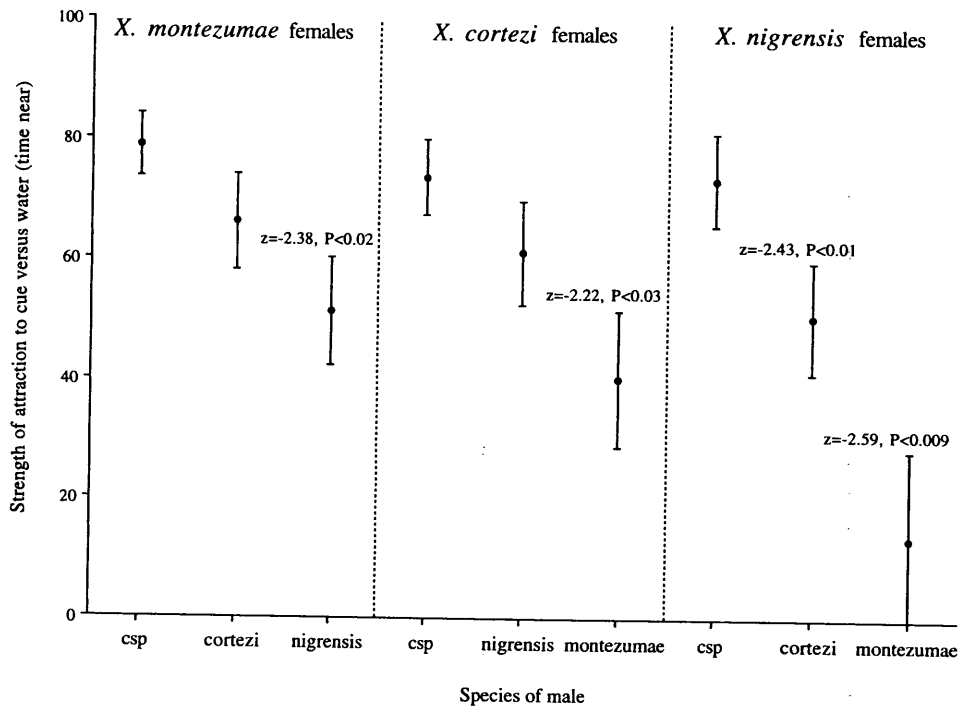

FIG. 2. Strength of attraction to the olfactory cue in cue versus control water trials for three species of northern swordtails. Csp, conspecific. Significant differences are noted between the females' response to the conspecific and the particular heterospecific cue. Data for *Xiphophorus cortezi* are from McLennan and Ryan (1997).

Table 1. Extended.

Xiphophorus nigrensis females (n = 16)		
Total time (sec)	% time near	% time interacting
144.3 (69.7)	36.0 (21.4)	12.2 (15.2)
145.5 (68.6)	34.3 (23.9)	11.6 (12.4)
ns (P = 0.99)	ns (P = 0.64)	ns (P = 0.68)
140.7 (49.7)	36.3 (18.7)	13.0 (11.2)
142.5 (48.6)	37.1 (20.9)	14.4 (13.0)
ns (P = 0.59)	ns (P = 0.67)	ns (P = 0.70)
52.7 (42.0)	27.1 (28.3)	6.9 (11.6)
247.3 (42.0)	65.5 (22.0)	31.9 (20.7)
$z = -3.52, P < 0.0004$	$z = -2.95, P < 0.004$	$z = -3.46, P < 0.0005$
85.2 (51.6)	35.6 (22.1)	10.3 (8.2)
214.8 (51.6)	64.2 (19.4)	32.0 (21.0)
$z = -3.36, P < 0.0008$	$z = -3.10, P < 0.002$	$z = -3.05, P < 0.003$
134.7 (64.7)	39.2 (30.0)	16.5 (18.3)
165.3 (64.7)	48.8 (20.0)	20.3 (16.1)
ns (P = 0.53)	ns (P = 0.44)	ns (P = 0.50)

alternative, and even preferred each other's males to *X. montezumae*. From a phylogenetic perspective, this implies that *X. cortezi* and *X. nigrensis* share an apomorphic change in some aspect of their olfactory cue-receiver system that is not shared with *X. montezumae*. The interaction between these two species, however, is not symmetrical, even though they are so closely related. Although *X. cortezi* females responded as strongly to the olfactory cues from *X. nigrensis* males as they did to the scent of their own males, the reverse was not true (McLennan and Ryan 1997; Fig. 2). Not surprisingly,

given this asymmetry, *X. cortezi* females made mistakes in the mate discrimination trials, whereas *X. nigrensis* females' preference for their own males was absolute (Fig. 3). These results imply that there is something derived in the olfactory cue-receiver system of *X. nigrensis* compared to *X. cortezi*.

Xiphophorus montezumae females are as strongly attracted to *X. cortezi* as they are to their own males. The strength of this attraction has an impact on interspecific mate choice: When offered the scent of *X. cortezi* males as the alternative, *X. montezumae* females do not show a strong preference for
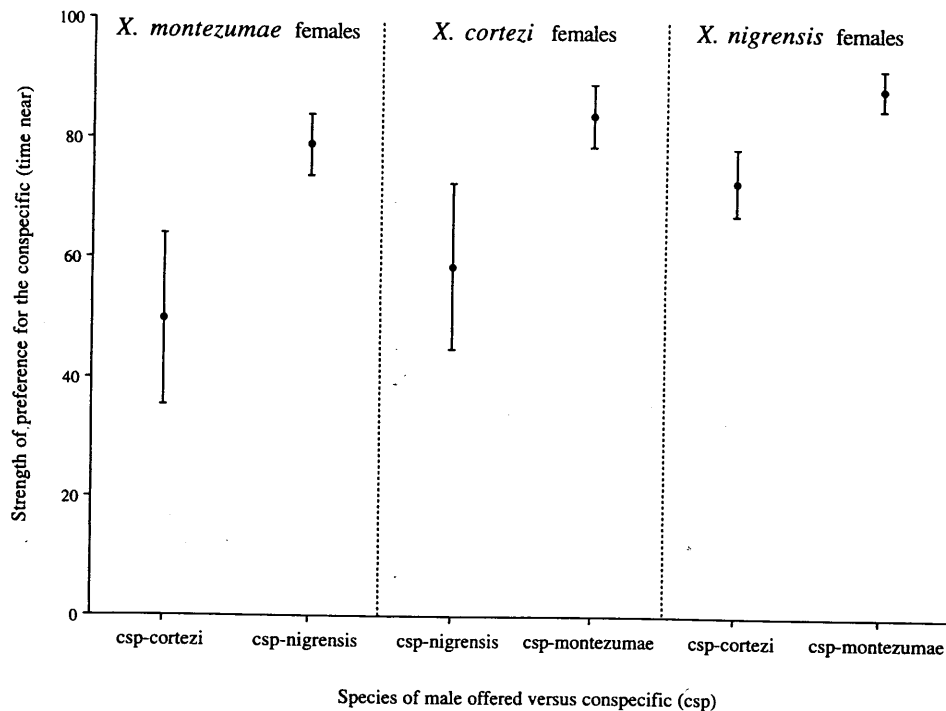

Fig. 3. Strength of preference for the conspecific in conspecific versus heterospecific choice trials for three species of northern swordtails. Data for *Xiphophorus cortezi* are from McLennan and Ryan (1997).

TABLE 2. Mean results (SD) of interspecific mate choice tests. *, test retains its significance after the sequential Bonferroni correction.

	Xiphophorus montezumae females (n = 16)			Xiphophorus nigrensis females (n = 16)		
	Total time (sec)	% time near	% time interacting	Total time (sec)	% time near	% time interacting
Conspecific versus heterospecific 1[a]	215.6 (74.7) / 84.4 (74.7) / $z = -2.43, P < 0.02$	73.0 (16.0) / 56.9 (24.7) / $z = -2.17, P < 0.03$	33.5 (19.1) / 17.9 (21.7) / $z = -1.96, P < 0.05$	232.6 (36.5) / 67.4 (36.5) / $z = -3.52, P < 0.0004$*	63.6 (16.8) / 29.3 (17.7) / $z = -3.52, P < 0.0004$*	33.4 (15.2) / 6.1 (8.4) / $z = -3.31, P < 0.0009$*
Conspecific versus heterospecific 2[b]	257.8 (33.1) / 42.2 (33.1) / $z = -3.52, P < 0.0004$*	72.8 (15.3) / 33.9 (27.9) / $z = -3.46, P < 0.0005$*	34.1 (27.6) / 6.8 (8.7) / $z = -3.05, P < 0.003$*	258.3 (33.2) / 41.7 (33.2) / $z = -3.52, P < 0.0004$*	74.6 (18.5) / 19.4 (11.0) / $z = -3.52, P < 0.0004$*	35.7 (23.3) / 1.6 (2.3) / $z = -3.41, P < 0.0007$*
Heterospecific 1 versus heterospecific 2	187.6 (55.8) / 112.5 (56.0) / $z = -2.38, P < 0.02$	63.0 (23.1) / 62.5 (22.4) / ns, $P = 0.92$	23.9 (23.1) / 12.0 (11.2) / $z = -2.16, P < 0.04$	198.4 (62.0) / 101.6 (62.0) / $z = -2.58, P < 0.01$*	56.9 (24.5) / 38.7 (28.2) / ns, $P = 0.12$	23.0 (25.6) / 10.3 (10.2) / ns, $P = 0.26$

[a] For both X. montezumae and X. nigrensis females, heterospecific 1 = X. cortezi males.
[b] For X. montezumae females, heterospecific 2 = X. nigrensis males; for X. nigrensis females, heterospecific 2 = X. montezumae males.

their own males. Indeed, three of 16 females actually preferred the scent of X. cortezi, and two females responded equally to both cues (did not choose). Olfactory cues have traditionally been classified as long-distance signals in moving water (Dusenbery 1992; Weaver and Atema 1998), allowing a female to detect the "I am here" message from the male before she can actually see him. Studies of interactions between temporally displaced signals indicate that the first cue (in this case chemical) functions to alert the receiver to the presence of the second cue (visual), increasing the probability of its detection and recognition (Endler 1992, 1993; Wiley 1994). Olfactory cues thus represent the first level of mate choice, allowing the female to recognize a potential mate (male swordtail). Once the female is closer to the male, visual cues become important. There is some evidence for intraspecific mate discrimination on the basis of body size/presence of courtship (X. nigrensis: Ryan and Wagner 1987) and the length of either the caudal sword (X. helleri: Basolo 1990a,b) or the caudal fin (X. variatus: Halnes and Gould 1994). Xiphophorus montezumae males are the largest and have the longest sword length/standard length index of all the northern swordtails (Rauchenberger et al. 1990). It is thus possible that any mistakes that might occur based upon the female's initial response to the olfactory cues would be overridden by her response to visual cues if both conspecific and heterospecific males are available for comparison. However, if an X. montezumae female were to encounter an X. cortezi male on his own, it is possible that she might make a mating mistake based on her attraction to the olfactory cue. At the moment these two species are not sympatric, but the results of this study sound a warning for the consequences of man-made sympatry between them.

Xiphophorus montezumae females show a significantly weaker response to X. nigrensis than to their own males or to X. cortezi. Interpretation of these results in a phylogenetic context is confounded by the different tree topologies for the northern swordtails; X. montezumae is either separated from X. cortezi by five nodes and from X. nigrensis by six nodes (Rauchenberger et al. 1990) or is equidistant from the two species (Meyer et al. 1994). Although there is ambiguity about the number of speciation events between X. montezumae and X. nigrensis, both tree topologies do support the hypothesis that the olfactory cue of X. nigrensis has diverged compared to X. cortezi from the perspective of X. montezumae females. That divergence has been strong enough to create an asymmetry in the strength of interspecific attraction between X. montezumae and X. nigrensis. Female X. montezumae are more strongly attracted to the olfactory cue from X. nigrensis males than vice versa (Mann Whitney U-test: total time: $z = -2.07, P < 0.04$; time interacting: $z = -2.83, P < 0.005$; time near: $z = -1.96, P < 0.05$). Given that all species responded in a similar way to the scent of their own males (Kruskal-Wallis test: total time: $H = 1.69, P < 0.43$; time interacting $H = 0.90, P < 0.64$; time near: $H = 3.35, P < 0.19$), it is unlikely that this result reflects a difference in female mating propensity among the species (Bateman 1949; Barton and Charlesworth 1984), but rather is additional evidence for the derived nature of the X. nigrensis olfactory cue-receiver system.

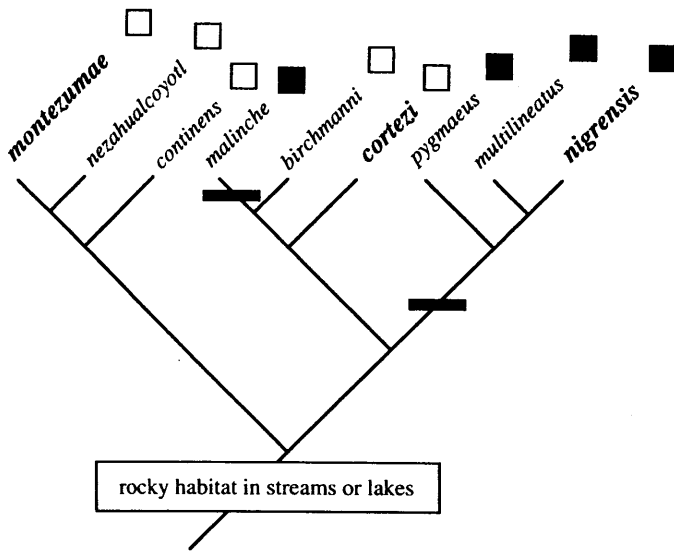The most obvious difference among the three species is

FIG. 4. Habitat type optimized onto the Rauchenberger et al. (1990) tree for the northern swordtails. White box, rocky habitat in streams or lakes; black box, sandy or muddy bottomed stream with submerged vegetation. Similar conclusions about the plesiomorphic and derived states are reached when the data are optimized onto the Meyer et al. (1994) tree.

the size of their geographic ranges. *Xiphophorus cortezi* and *X. montezumae* are both widespread species postulated to have arisen via vicariant speciation. *Xiphophorus nigrensis*, on the other hand, has a very limited range and is not currently sympatric with any other members of the northern swordtail clade. In other words, *X. nigrensis* appears to be a peripheral isolate (Rauchenberger et al. 1990). Peripheral isolates speciation is often associated with rapid evolutionary change in response to directional selection in a new environment (Mayr 1963; Wiley 1981; Brooks and McLennan 1991; Whitlock 1997). Five of the nine northern swordtail species prefer rocky habitats in springs and streams, whereas the other four species, including *X. nigrensis*, favor streams with "sandy to muddy bottoms and extensive stands of submerged aquatic vegetation" (Rauchenberger et al. 1990, p. 28). Optimization of the habitat data onto the phylogenetic trees for the northern swordtails indicates that the rocky habitat is plesiomorphic, whereas sandy/muddy bottom plus submerged vegetation is derived (Fig. 4). The efficacy of chemical transmission in water is dependent upon a variety of factors, including presence of other solutes and water temperature. It is thus possible that different transmission parameters, and therefore a different selective regime, in the new environment influenced the evolution of the olfactory cue in *X. nigrensis* (Endler 1992, 1993; see also Ryan et al. 1990). If gene flow between the peripheral isolate and the remainder of the ancestral stock was disrupted following geographical isolation, then effects of the new selective regime would be further enhanced (García-Ramos and Kirkpatrick 1997). Taken together, these factors may explain why *X. nigrensis* is more reproductively isolated based on olfactory cues than are either *X. cortezi* or *X. montezumae*. One possible way to examine this suggestion further would be to gather information about the olfactory recognition-discrimination abilities of *X. multilineatus*, the

sister species to *X. nigrensis* and itself a peripheral isolate in the derived habitat.

Clearly, olfactory cues provide species-specific information and are thus a cohesive component (Wiley and Brooks 1982; Hull 1984; Brooks and Wiley 1988; Templeton 1989) of the mate recognition system (sensu Paterson 1985) in these fish. Just as clearly, that information is not absolute for all species: *X. nigrensis* is the most reproductively isolated, while *X. montezumae* is the most likely to make mating mistakes based on olfactory cues alone (Fig. 3). During the course of this experiment, tanks were mistakenly repaired twice in the stock room where the test females were being kept. Acetic acid from the silicone sealant flooded the room and decreased the pH of the water in test female jars. In both cases, the females stopped responding to the olfactory cue within 12 h and the experiments had to be terminated. We have no explanation for this observation. We mention it only to indicate that changes in water quality may have a devastating effect on the breeding system of these fishes, even when the fish themselves look perfectly healthy. The planet is currently in the midst of a biodiversity crisis. Understanding the ways in which that biodiversity was produced in the first place, and the role of mate choice in that production, is critical to understanding how to solve this crisis.

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