

Mate recognition systems in a species flock of Mexican pupfish

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Keywords:

chemical cues;
Cyprinodon;
premating isolating mechanisms;
speciation;
visual cues.

Abstract

The pupfishes of Lake Chichancanab (Mexico) provide a unique opportunity to study the evolution of premating reproductive barriers. This group of endemic species is of recent origin and the lineages differ in morphological traits indicating trophic specialization, but show very little genetic change. We studied the function of visual and chemical cues in the mate recognition system of three morphospecies, *Cyprinodon beltrani*, *C. labiosus* and *C. maya*. Only in *C. maya*, which is genetically the most distinct member of the group, do females show the ability to identify males of their own type, both visually and chemically. *C. labiosus* females discriminated between their own and *C. beltrani* males based on chemical but not visual cues. *C. beltrani*, which is morphologically the least differentiated member of the group, showed no interspecific mate recognition. Females responded more strongly to the visual and chemical cues of their own males than to their own females, but they did not discriminate between their own males and those of the other two morphospecies based upon either type of cue. These results suggest that different levels of behavioural isolation have been established: most isolated is *C. maya*, and the least is *C. beltrani*. *C. labiosus* has an intermediate position. The results also suggest that species recognition cues based on different sensory modalities do not necessarily evolve together, and may evolve at different rates.

Introduction

Mating discrimination is the ability of individuals of one sex to discriminate among and selectively mate with conspecific members of the opposite sex (Andersson, 1994; Pfennig, 1998). Species-specific characters which serve in the recognition of mates tend to be better developed in sympatric than in allopatric species (e.g. Howard, 1993). Mate recognition systems are often based on several sensory modalities. Studies of mate choice have focused either on the kinds and functions of traits that serve as the basis for intraspecific mate choice and sexual selection, or on the nature of cues that promote conspecific mating in zones of secondary contact

between previously allopatric species (Wasserman & Koepfer, 1977; McKinnon & Liley, 1987; Ryan, 1994). In most cases females tend to be the discriminating sex, since the costs of producing offspring of lower fitness are greater for females than they are for males (Trivers, 1972). In fishes, acoustic, electromagnetic, tactile, visual and chemical cues are used to identify sexual partners (Kramer, 1990; Hawkins, 1993; Hara, 1994; McPhail, 1994). Although several studies have focused on the role of visual cues in species recognition (reviewed in Andersson, 1994), chemical cues have been studied only in a few groups of fishes, including poeciliids (Liley, 1966; Crapon de Caprona & Ryan, 1990; McLennan & Ryan, 1997), cyprinids (Bloom & Perlmutter, 1978), gouramis (McKinnon & Liley, 1987) and catfish (Rubec, 1979).

We studied the function of chemical and visual cues in the mate recognition system in an endemic species flock of pupfishes (*Cyprinodon* spp.) in the Laguna Chichancanab, Yucatan, Mexico. Five species were described based

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on differences in morphological characters, especially in the structure of their feeding apparatus (Humphries & Miller, 1981; Humphries, 1984a). All five morphospecies co-occur in the same habitats and are dispersed throughout the lake (Humphries & Miller, 1981; Humphries, 1984a). Physical factors, such as the small size and shallowness of the lake, and rather homogenous abiotic conditions favour ubiquitous use of habitats by all five morphospecies. Both geological and molecular data indicate that the pupfish flock is of recent origin. The lake dried out 8000 years ago and since then the water level has been relatively stable (Covich & Stuver, 1974; Hodell *et al.*, 1995). Thus colonization and subsequent differentiation of the group have occurred since that time. Genetic divergence in this group is low. Allozyme studies have shown a high degree of genetic similarity between morphospecies (the range of Nei's index is 0.972–1.00), and is more typical of the variation found within a species (Humphries, 1984b). Sequences of the D-loop of mtDNA have shown that the haplotypes found in four of these five putative species have a high degree of overlap (80%). Only *C. maya* has a unique haplotype and is assumed to be reproductively isolated (Strecker *et al.*, 1996). Occurrence of fish with intermediate morphologies suggests that hybridization still occurs, at least between some of these morphospecies (Humphries & Miller, 1981; Strecker *et al.*, 1996). Phylogenetic relationships between these morphospecies cannot be resolved with current data. The available evidence points to a monophyletic group which seems to be in the process of speciation (Strecker *et al.*, 1996). Consequently, divergence from the common ancestor began after the post-Pleistocene desiccation of the lake and is still in progress. This system is ideal to study traits that function in mate recognition for the following reasons: (1) the small number of morphospecies, (2) the co-occurrence of all morphospecies and (3) the recent origin of the species flock.

We performed female choice tests on three of the five morphospecies, *C. beltrani*, *C. labiosus* and *C. maya*, to address the following questions: (1) Can females discriminate between sexes? (2) Do females discriminate between their own males and those of the other morphospecies? (3) Are chemical and/or visual cues important in mate recognition and function as potential species reproductive barriers?

We chose these three morphospecies for the following reasons. *C. beltrani* is morphologically and ecologically similar to the sister species *C. artifrons* which today occurs in coastal Yucatan habitats, and feeds like most pupfish on detritus and algae. Therefore, it is assumed to be the basal member of the flock. *C. labiosus*, characterized by a distinctive mouth and fleshy lips, is similar in size to *C. beltrani* (22–37 mm standard length) but differs from *C. beltrani* in many shape-related features (Humphries & Miller, 1981). *C. maya* is the largest member of the group (30–85 mm standard length) and genetically the most

distinct morphospecies in the lake. *C. labiosus* and *C. maya* are both carnivorous.

Pupfish have a polygynous breeding system. Males develop nuptial coloration when they establish breeding territories over suitable spawning substrate (Kodric-Brown, 1977). Females choose males and spawn with them within the territories. In the Laguna Chichancanab reproduction occurs throughout the year. The breeding coloration of *C. labiosus* and *C. beltrani* males is nearly identical, with a black body and fins, metallic blue shoulder and yellow caudal peduncle. *C. maya* males show a less intense breeding colouration and a pattern of distinct vertical bars. *C. labiosus* and *C. beltrani* defend discrete territories over similar substrates, while *C. maya* appear to be less territorial. Females of all three morphospecies show the typical cryptic colour patterns of buff background with brown vertical bars characteristic of the genus.

Methods

Fish used in these experiments were the first generation offspring of individuals caught in the wild. Each laboratory stock started with 20 individuals of both sexes. Fish were kept in stock tanks separated by morphospecies at a temperature of 25–27 °C, salinity of 5‰ and a 12-h light–dark cycle. They were fed *ad libitum* with Tetramin dry food, supplemented with live cladocerans, *Tubifex* and *Artemia*. All fish used in the experiments were experienced. Choice tests were performed with three or four stimulus fish to ensure that males show courtship behaviour and develop coloration during the experiment. Every test female was used only once per experiment. Sample sizes of fish used in the experiments differed because of availability of adult gravid females and the number of individuals that had to be discarded because they failed to respond appropriately to test stimuli. *n* refers to the scored females tested. Since the data on female responses were not normally distributed, they were analysed with Wilcoxon signed-ranks tests.

Visual cues

Two small water-tight tanks (20 cm × 30 cm × 23 cm) were placed within a larger tank (92 cm × 61 cm × 23 cm), eliminating the transmission of chemical cues. Four individuals of the same sex were introduced into each of the smaller tanks to act as stimulus fish. The fish were allowed to acclimatize overnight. A test female was introduced into the large experimental tank, and if she was actively swimming, her movements were observed for a 10-min period. Female preference was measured as the amount of time she swam in contact with the glass sides of the small tanks. Trials in which the female spent less than 3 min with the stimulus fish were discarded. If males were the stimulus fish, trials were started only if they courted. We used two different sets of

stimulus fish for the male and the female experiments each. The sets were changed after two experimental days. Except in the experiments with the larger species *C. maya*, stimulus fish were carefully matched for standard length. To control for a side bias, we switched the position of the stimulus fish and tested the female on the following day.

Females of *C. beltrani* and *C. labiosus* were examined both with males and females of their own and with the other morphospecies. When using females as stimulus fish for *C. beltrani* and *C. labiosus* four and two females, respectively, were discarded because they spent less than 3 min with the stimulus fish. Seven *C. beltrani* females were discarded when using males as stimulus fish. To test the ability of the females to discriminate between sexes we compared the time eight females of each morpho-species spent with stimulus males and females. Additionally, we tested the preferences of *C. beltrani* and *C. maya* females for their own and heterospecific males. In this case we used three stimulus fish per tank since *C. maya* males are considerably larger. In these experiments we discarded four *C. beltrani* and seven *C. maya* females because they spent less than 3 min with the stimulus fish.

Chemical cues

The experimental design was similar to the one of Crapon de Caprona & Ryan (1990). We used a 100-L experimental tank (100 cm × 35 cm × 35 cm) filled up to 80% with water. Two vertical lines drawn on the front of the tank divided it into three compartments of equal size. Two small tanks, containing 4 L of the test water or control water, were placed over this experimental tank. Plastic hoses fed the water from the small tanks into the two outer compartments of the experimental tank. To avoid mixing of the test water during the trial, we adjusted both the height of inflow, to 10 cm below the water surface, and the amount of test water introduced into the experimental tank. The inflow rate of test water was 2.300 ± 200 mL per 10 min. The test water was taken from 50-L tanks containing either four males or four females of a morphospecies which remained for 24 ± 2 h in these tanks without feeding to avoid food contamination. The control water was taken from a 50-L tank without fish. After every trial the water in the 50-L tanks was replenished, the experimental apparatus was thoroughly cleaned and the experimental tank was refilled.

A test female was introduced into the experimental tank. The inflow of the test water started when the female stayed in the middle compartment. The observation period began after the female swam a distance of one body length, and lasted 10 min. We measured the time the female spent in each of the outer compartments of the experimental tank. To test for a side preference the trial was repeated and the test stimuli were switched. We

randomized the presentation of the stimuli. The trials were run on two consecutive days.

C. beltrani females were tested with water from their own males or females and control water. *C. beltrani* females also were tested with their own males and either *C. labiosus* or *C. maya* males. The *C. labiosus* and *C. maya* females were tested with their own and *C. beltrani* males.

Results

Visual cues

C. beltrani females did not discriminate between their own and *C. labiosus* females ($P = 0.3905$, $n = 20$). Neither did they discriminate between their own and *C. labiosus* males ($P = 0.2043$, $n = 20$; Fig. 1A; Table 1) or their own and *C. maya* males ($P = 0.444$, $n = 20$; Fig. 1B; Table 1). They did, however, spend less time associating with stimulus females than with stimulus males ($P = 0.0117$, $n = 8$). Bonferroni adjustment shows that one sample is significantly different.

Table 1 Differences in the time (min) test females spent with their own males and with males of the other morphospecies during visual trials. Numbers represent time with own males minus time with the other males.

<i>C. beltrani</i> females with:		<i>C. labiosus</i> females with:	<i>C. maya</i> females with:
<i>C. labiosus</i> males	<i>C. maya</i> males	<i>C. beltrani</i> males	<i>C. beltrani</i> males
-5.50	-7.02	-7.27	0.93
-3.94	-5.17	-5.75	1.12
-2.24	-4.08	-4.98	1.67
-1.42	-4.07	-4.10	1.78
-1.41	-2.72	-4.09	3.09
-0.11	-2.20	-3.74	4.02
0.02	-1.79	-3.24	4.72
0.14	-1.17	-2.75	4.99
0.15	-0.68	-2.44	
0.25	-0.51	-2.00	
0.32	-0.38	-0.97	
0.53	0.02	-0.70	
0.62	0.04	0.24	
1.30	0.68	0.27	
1.34	1.09	0.50	
3.90	1.64	0.79	
3.95	1.76	1.08	
5.32	3.24	1.65	
5.52	4.61	1.65	
7.32	6.25	2.06	
	2.75		
	6.20		
	6.49		
	9.57		

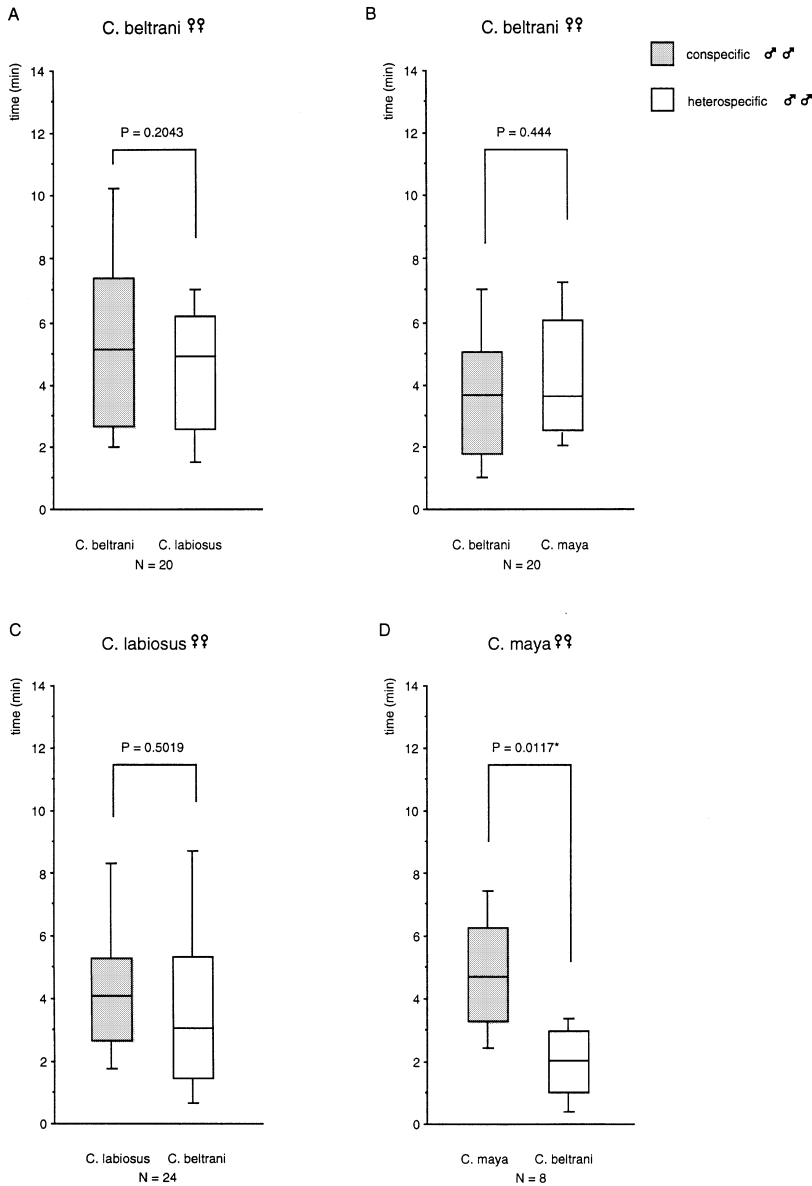


Fig. 1 Time females spent with conspecific and heterospecific males during the visual trials. (A,B) *C. beltrani*, (C) *C. labiosus*, (D) *C. maya*. *n* = number of females tested. Horizontal lines show the median, boxes represent quartiles and vertical lines range from the 10th to the 90th percentile.

C. labiosus females did not show a preference when given a choice between their own and *C. beltrani* females ($P = 0.6012$, $n = 20$) or their own and *C. beltrani* males ($P = 0.5019$, $n = 24$; Fig. 1C; Table 1). They spent more time with males than with females ($P = 0.0117$, $n = 8$). Bonferroni adjustment shows that one sample is significantly different.

Unlike the two other morphospecies, *C. maya* females showed a significant preference for their own males when given a choice between their own and *C. beltrani* males ($P = 0.0117$, $n = 8$; Fig. 1D; Table 1). All of the females tested preferred conspecific males.

Chemical cues

C. beltrani females preferred the water from their own males over the control ($P = 0.0128$, $n = 11$), but did not discriminate between test water from conspecific females and control ($P = 0.2026$, $n = 10$). *C. beltrani* females did not show a preference for their own males over either *C. labiosus* males ($P = 0.5937$, $n = 11$; Fig. 2A; Table 2) or *C. maya* males ($P = 0.8589$, $n = 11$; Fig. 1B; Table 2). In contrast, *C. labiosus* females preferred their own males over *C. beltrani* males ($P = 0.0262$, $n = 11$; Fig. 1C; Table 2). *C. maya* females expressed a strong preference for conspecific males over

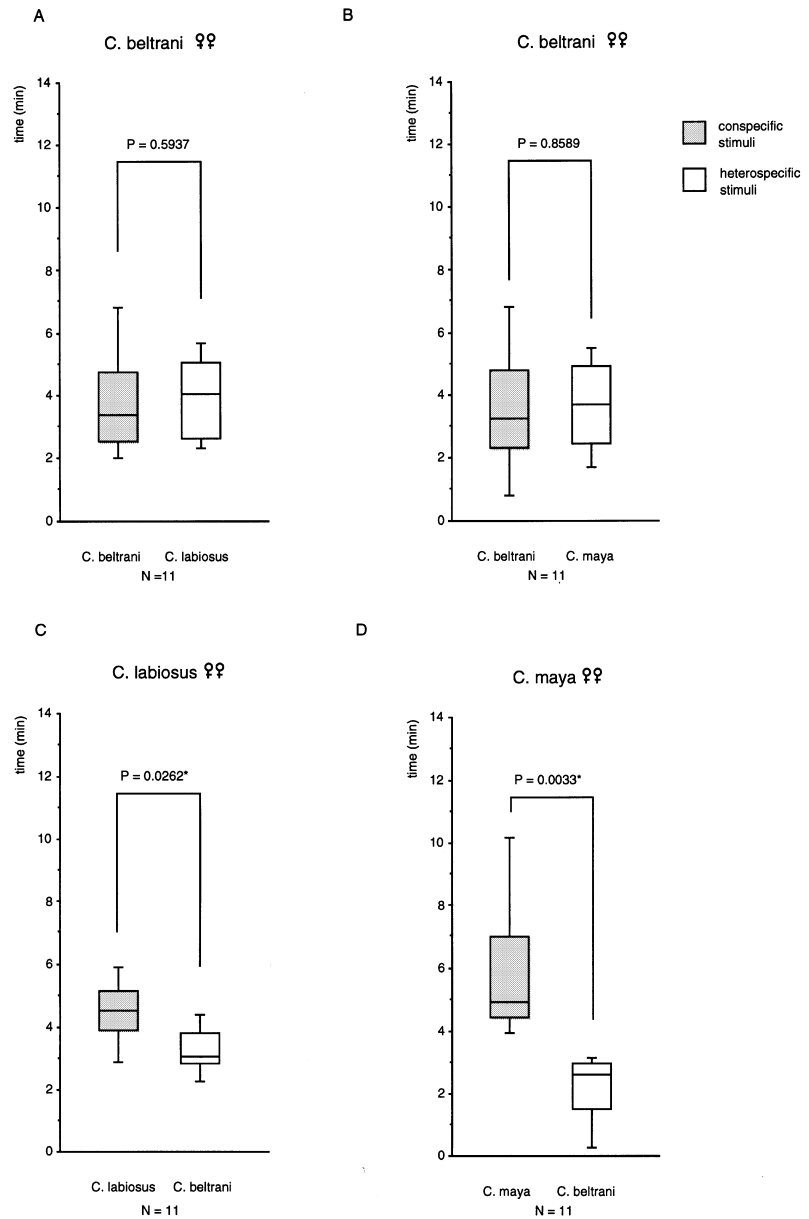


Fig. 2 Time females spent at each side of the test aquarium when presented with chemical stimuli of conspecific or heterospecific males. (A,B) *C. beltrani*, (C) *C. labiosus*, (D) *C. maya*. n = number of females tested.

C. beltrani males ($P = 0.0033$, $n = 11$; Fig. 1D; Table 2). In this case every female tested preferred males of her own morphospecies.

Discussion

The results of our study indicate that the importance of visual and chemical cues in mate recognition varies among these three sympatric morphospecies of pupfish (Fig. 3). Females from the putatively most basal member of the group, *C. beltrani*, are the least choosy. They do not discriminate between conspecific and heterospecific males based upon either visual or olfactory cues.

C. labiosus females also do not visually discriminate between their own males and *C. beltrani* males, but they do show a distinct preference for their own males when given the opportunity to use olfactory stimuli. *C. maya* females are capable of discriminating between their own males and *C. beltrani* males based upon both visual and chemical cues.

The inability of *C. beltrani* and *C. labiosus* females to discriminate visually between their own and those of the other morphospecies is in agreement with the observation that the nuptial colour of *C. beltrani* and *C. labiosus* males is similar. It suggests that selection for divergence of nuptial colours and patterns is not very strong or that

Table 2 Differences in the time (min) test females spent with the stimuli of their own males and with the stimuli of the males of the other morphospecies during chemical trials. Numbers represent time with stimuli of own males minus time with stimuli of the other males.

<i>C. beltrani</i> females with:		<i>C. labiosus</i> females with:	<i>C. maya</i> females with:
<i>C. labiosus</i> males	<i>C. maya</i> males	<i>C. beltrani</i> males	<i>C. beltrani</i> males
-7.91	-4.30	-2.10	0.20
-6.45	-2.79	-1.19	1.98
-3.72	-2.73	1.28	2.25
-0.28	-2.03	1.35	3.33
0.10	-1.23	1.73	3.44
0.67	-0.95	1.93	3.56
4.15	1.56	1.93	5.50
4.65	3.52	3.19	7.41
4.89	5.84	3.82	7.67
5.35	7.20	4.74	12.18
5.95		5.11	16.40

selection is opposed by extensive gene flow for at least these two members of the group. In *C. maya*, however, there has been sufficient divergence, both in male traits and in female preferences for them, to establish a premating reproductive barrier based on visual cues. While visual signals are important in mate recognition, the specific cues used, whether size, colour pattern or some other male trait, remain to be determined.

Differences in chemical cues may have originated, at least in part, from differences in diet due to exploitation of different trophic resources (McKinnon & Liley, 1987). Thus the origin of interspecific mate recognition based upon chemical cues may have been associated with a change in diet from detritus and algae to carnivory. Since all fish were fed the same diet in the laboratory, chemical cues probably are not directly functionally correlated with trophic specialization (Turner & Grosse, 1980; Liley, 1982). It is likely that observed differences in the production and perception of chemical cues by *C. maya*, and to a lesser extent by *C. labiosus*, have a genetic basis.

Our data indicate that mate recognition based upon olfactory cues seems to be evolving at a slightly faster rate than mate recognition based on visual cues. Of the three morphospecies, only *C. maya* has a mate recognition system based on both visual and olfactory modalities. *C. maya* is also genetically the most differentiated member of the species flock; it is the only one with a unique haplotype and reproductive isolation is assumed (Strecker *et al.*, 1996). *C. labiosus* shows a less well developed mate recognition system that uses only olfactory cues. *C. beltrani*, the putatively basal member of the species flock, lacks the ability to discriminate between conspecifics and heterospecifics based both on visual and olfactory cues. The incomplete reproductive isolation of *C. labiosus* and *C. beltrani*, based on the two sensory cues

we tested them for, is consistent with the results of the molecular analysis of the D-loop haplotypes which have shown that the two lineages are genetically indistinguishable (Strecker *et al.*, 1996). This is also consistent with results of mate choice experiments in which *C. labiosus* and *C. beltrani* females were allowed to spawn with males of their own and the other morphospecies (Strecker, 1996; Strecker & Kodric-Brown, unpublished observations). In these experiments, *C. beltrani* spawned as often with their own and with the other morphospecies, whereas *C. labiosus* females spawned more often (70%) with their own males. Additional evidence is the presence of naturally occurring hybrids in the lake (Humphries & Miller, 1981; Strecker, 1996).

A special form of frequency-dependent selection also may play a role in the evolution of the observed asymmetries in the mate recognition system of the three members of the pupfish species flock (Watanabe & Kawanishi, 1979; Wilson, 1989). *C. beltrani* is the most abundant morphospecies in the lake (68–85% captured in seine hauls; Humphries & Miller, 1981). If there is a cost of producing intermediate types (hybrids), this cost would be least for *C. beltrani*, because if females just chose males at random they would still mate with their own type most of the time. Thus *C. beltrani* should be under relatively low selective pressure to develop a premating reproductive barrier. The cost of random matings would be higher for the less abundant *C. labiosus* and *C. maya*. Since the territories of *C. labiosus* males often are interspersed among those of *C. beltrani* males, random matings would be especially costly for *C. labiosus* females.

Differences in levels of premating reproductive isolation may also reflect the intensity of disruptive selection. Disruptive selection can generate and maintain ecotypic adaptive divergence if that divergence is coupled with assortative mating, even under sympatric conditions (Bush, 1975; Endler, 1977; Rosenzweig, 1978; Rice, 1984). Trophic differentiation to exploit different food resources apparently plays a major role in the morphological and behavioural divergence of several sympatric fish species, e.g. in *Gasterosteus* (McPhail, 1984), *Gila* (Galat & Vucinich, 1983), *Coregonus* (Smith & Todd, 1984; Bernatchez *et al.*, 1996), *Osmerus* (Taylor & Bentzen, 1993) and *Ilyodon* (Turner & Grosse, 1980) and may be an important selective factor in the evolution of the pupfish of the Laguna Chichancanab as well. There are several examples in which such resource-based polymorphism leads to evolution of different morphotypes that may not be genetically distinct (e.g. Skúlason & Smith, 1995). Such polymorphism could be the initial phase in a speciation process (Maynard Smith, 1966; Wilson, 1989). In other cases, such as African cichlids, trophic differentiation and sexual selection have led to species that are reproductively isolated and genetically distinct (Fryer & Iles, 1972; Turner & Burrows, 1995; Galis & Metz, 1998).

Assortative mating, which is reinforced through natural selection against unfit hybrid offspring, may be an

Visual cues

Chemical cues

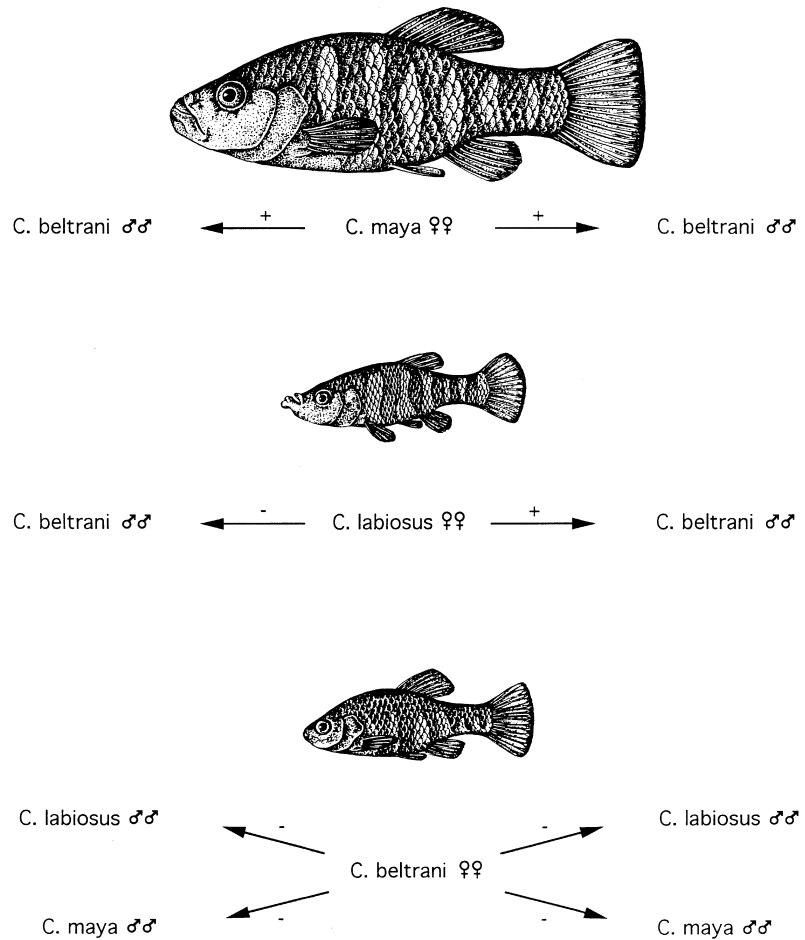


Fig. 3 Summary of responses of *C. beltrani*, *C. labiosus* and *C. maya* females presented with visual and chemical cues of conspecific and heterospecific males.

important selective force in sympatric speciation (Sætre *et al.*, 1997; Nagel & Schluter, 1998; Rundle & Schluter, 1998). Sexual selection, in the form of female preferences for specific male characters, can enhance reproductive isolation (Lande, 1982; West-Eberhard, 1983; Liou & Price, 1994; Payne & Krakauer, 1997). Hawaiian fruit flies (Dominey, 1984) and African haplochromine cichlid fishes (Fryer & Iles, 1972; McKaye, 1991; Turner & Burrows, 1995; Seehausen *et al.*, 1997) provide dramatic examples of the importance of sexual selection in sympatric speciation. It is assumed that two processes – sexual selection and disruptive selection – act together to produce and maintain species diversity (Galis & Metz, 1998).

In the pupfish of the Laguna Chichancanab it is difficult to determine the extent to which sexual selec-

tion contributes to the observed differences in the mate recognition system. Pupfish do not show a dramatic divergence in sexually selected traits such as nuptial coloration, and thus visual cues based on such differences cannot be very important. The role of sexual selection in the development of mate recognition systems based on chemical cues has yet to be determined for pupfish, although it is implicated in the reproductive isolation of other groups of fishes such as swordtails (McLennan & Ryan, 1997).

The recent origin of this *Cyprinodon* species flock is reflected by different stages of reproductive isolation, suggesting either that the process of speciation is an ongoing one or that this system is at an equilibrium and represents a stable polymorphism. Because the pupfishes of Laguna Chichancanab are at different stages of genetic

and morphological differentiation, they provide a unique opportunity to understand the evolutionary process of sympatric speciation.

Acknowledgments

We would like to thank J. H. Brown for comments on an earlier version. U.S. was supported by a grant from the Deutscher Akademischer Austauschdienst (DAAD). A.K.B. was supported by an NSF grant IBN-98-06483.

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Received 7 September 1998; revised 13 October 1998; accepted 24 February 1999