



Responses of *Cyprinodon maya* and *C. labiosus* females to visual and olfactory cues of conspecific and heterospecific males

ASTRID KODRIC-BROWN^{1*} and ULRIKE STRECKER²

¹Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA

²Zoologisches Institut und Zoologisches Museum der Universität Hamburg, Martin-Luther-King-Platz 3, D20146 Hamburg, Germany

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Premating isolating mechanisms, based on visual and olfactory cues, were studied in two members of a young species flock which is endemic to Lake Chichancanab in Mexico. The two species, *Cyprinodon maya* and *C. labiosus*, differ in morphological traits that may be associated with trophic differentiation, but show little genetic change. Female *C. maya* strongly preferred conspecific males over *C. labiosus* males, based on visual as well as olfactory cues. *C. labiosus* females discriminated between conspecific and heterospecific males based on olfactory cues but not visual ones. Asymmetries in the strength of female preferences for conspecific males in this species pair suggest that mate recognition systems based on visual and olfactory cues evolve at different rates and track the degree of genetic differentiation.

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ADDITIONAL KEY WORDS: premating isolating mechanisms – Cyprinodontidae – fish – sympatric speciation – evolution.

INTRODUCTION

Lineages that evolved in sympatry or in zones of secondary contact between populations that differentiated in allopatry (e.g. Echelle & Kornfield, 1984; Bush, 1994; Schliewen, Tautz & Paabo, 1994; Bernatchez *et al.*, 1996) are particularly well suited to study traits that function in species recognition and conspecific mating (e.g. Liley, 1966; Wassermann & Koepfer, 1977; McKinnon & Liley, 1987; McPhail, 1994). However, the underlying behavioral mechanisms and sensory modalities of importance in the evolution of premating isolating mechanisms in sympatry and after secondary contact of closely related congeners are, for the most part, unknown. In African cichlids visual signals seem to be important in lineage divergence in sympatry (Seehausen & van Alphen, 1998). Although the importance of other sensory modalities (chemical, electrical, and acoustic) in recognition of conspecifics and mate choice in fishes has been studied in some groups (Stacey, Kyle & Liley, 1986; Hara, 1994; Kramer, 1990;

Hawkins, 1993), their role in sympatric speciation has yet to be demonstrated. For example, odours appear to function in mate recognition in several groups of fishes, among them poeciliids (Liley, 1966), belontiids (Lee & Ingersoll, 1979; McKinnon & Liley, 1987), cyprinids (Bloom & Perlmutter, 1978), swordtails (Crapon de Caprona & Ryan, 1990; McLennan & Ryan, 1997, 1999), and Mexican cyprinodontids (Strecker, 1996; Strecker & Kodric-Brown, 1999), but only in a few of these groups have they been shown to function in differentiating between conspecifics and heterospecifics. Even less is known about the use of multiple sensory modalities to identify conspecifics and to discriminate among potential mates (Crapon de Caprona & Ryan, 1990; Strecker, 1996; Strecker & Kodric-Brown, 1999). In studies of Northern swordtails, one of the few systems in which both visual and chemical cues and their potential function in recognition of conspecifics were investigated, chemical cues were of secondary importance to visual cues (McLennan & Ryan, 1997, 1999).

Our studies on mate recognition systems in a species flock of pupfishes provide insights not only into the relative importance of chemical and visual cues in

* Corresponding author. E-mail: kodric@unm.edu

discrimination between conspecific and heterospecific males, but also suggest that in this case selection on the chemical modalities is stronger than on visual ones.

The species flock of pupfishes (*Cyprinodon*) inhabiting the Laguna Chichancanab, Yucatan, Mexico consists of five species, described by Humphries & Miller (1981) and Humphries (1984a). Species differ in morphological traits, especially in the structure of the head and mouthparts. They inhabit a shallow lake with a homogeneous substrate, and all five species co-occur throughout the lake and occupy all habitats (Humphries, 1984a; Humphries & Miller, 1981). The geologic history of the Laguna Chichancanab suggests that it was dry 8000 years BP, but since then the water level has been relatively stable (Covich & Stuiver, 1974; Hodell, Curtis & Brenner, 1995). Thus geological dating provides a time frame for the colonization of the lake by pupfish and the initiation of sympatric differentiation. As expected, because of its recent origin, the pupfish flock shows little genetic differentiation based on allozyme and mitochondrial markers (Humphries, 1984b; Strecker *et al.*, 1996). Analysis of the D loop of mtDNA shows that one species (*C. maya*) has a unique haplotype suggesting reproductive isolation; in the other four species the mtDNA haplotypes overlap (Strecker *et al.*, 1996). Thus phylogenetic relationships between the pupfish species in Lake Chichancanab remain unresolved. The molecular data suggest that this group of pupfishes is of monophyletic origin and is still in the process of speciating. This is inferred from the recent origin of the group, the high degree of genetic overlap between members of the flock, and the presence of fish with intermediate morphologies, suggesting that pre-mating isolating mechanisms are still evolving. The pupfishes of lake Chichancanab are therefore ideally suited to look for the presence of pre-mating isolating mechanisms, and to determine the degree to which they are expressed among members of this group. In a previous study of the responses of *C. maya* and *C. beltrani* females to visual and chemical cues of conspecific and heterospecific males, it was apparent that *C. maya* was reproductively isolated from *C. beltrani*, whereas *C. beltrani* females were indiscriminate in their choice of conspecific and heterospecific males based on either visual or chemical cues. Species recognition was incomplete and asymmetrical between the *C. labiosus* and *C. beltrani* pair. *C. labiosus* females preferred conspecific males, based solely on chemical cues, whereas *C. beltrani* females were indiscriminate in their choice of males (Strecker, 1996; Strecker & Kodric-Brown, 1999, 2000).

In this study we examined the responses of *C. maya* and *C. labiosus* females to the visual and olfactory cues of their own and heterospecific males. We addressed the

following questions. Do females respond to the cues provided by heterospecific males? Do females discriminate between the cues of conspecific and heterospecific males? The two species differ in size, and to a certain extent in diet, although both are carnivorous (Stevenson, 1992). *C. maya* is larger (30–85 mm standard length) than *C. labiosus* (27–37 mm SL). *C. labiosus* has the fleshy lips and elongated head and mouth characteristic of a diet consisting of invertebrates, particularly snails (Humphries & Miller, 1981). The Chichancanab pupfishes have a polygynous mating system typical of pupfishes studied to date (Barlow, 1961; Kodric-Brown, 1977; Itzkowitz, 1978, 1979; Strecker, 1996). Males defend territories over suitable oviposition substrates such as rocky outcrops, submerged aquatic vegetation or even silt (Strecker, 1996). Gravid females visit these territories and spawn with the territorial male. Courtship and spawning behaviour of the three species of Chichancanab pupfishes studied to date is similar to that of other species of pupfish (Itzkowitz, 1978, 1979; Kodric-Brown, 1983). Humphries & Miller (1981) described the breeding coloration of males of the five species. Males develop a nuptial coloration characterized by a black body and fins, a metallic blue nape, and a yellow caudal peduncle and tail. *C. maya* males usually tend to have a less intense breeding coloration than *C. labiosus* males. *C. labiosus* males defend discrete breeding territories in shallow areas near the shoreline, whereas the territories of *C. maya* males are less well defined and much larger (Strecker, 1996; Kodric-Brown, personal observation). Both *C. maya* and *C. labiosus* females are cryptic with a pattern of olive-brown vertical bars on a buff background. Both species breed throughout the year and utilize similar breeding substrates and habitats (Strecker, 1996).

MATERIAL AND METHODS

MAINTENANCE OF FISH

Fish used in the experiments were the offspring of the first generation of laboratory-reared fish from parents caught in the wild. There were several aquaria of each species. Aquaria were kept at a temperature of 25–27 °C, salinity of 5 ppt, and a 12 h light-dark cycle. Fish were fed ad libitum with Tetramin dry food, supplemented with live cladocerans, *Tubifex* and *Artemia*. To eliminate potential biases due to familiarity with known conspecifics, test fish and stimulus fish came from different stock aquaria. Thus all test fish had no prior experience with the stimulus fish. For both visual and chemical trials we used groups of four males in order to ensure that the males engaged in courtship behaviour, developed breeding coloration and to minimize the probability that females were responding to individual rather than species-specific

characteristics of males. The experimental design for both experiments was similar to that described in Strecker (1996) and Strecker & Kodric-Brown (1999).

VISUAL CUES

Two small water-tight tanks (20 cm × 30 cm × 23 cm) were placed within a large one (92 cm × 61 cm × 23 cm), eliminating the transmission of chemical cues. The large tank was evenly illuminated with overhead lights (GE 'Plant and Aquarium' F20T12-PI/AQ). The two small aquaria were placed equidistant from the front and back and 15 cm from the sides of the large aquarium, to provide easy access to the test female to all sides of the two small aquaria. Four males of *C. maya* were introduced into one small aquarium and four males of *C. labiosus* were introduced into the other small aquarium. These males were allowed to acclimate overnight. A test female, either *C. maya* or *C. labiosus*, was removed from one of the stock aquaria and introduced into the large experimental aquarium. During an acclimation period which lasted up to 10 min, we observed the female. Once she was actively swimming, we started the 10-min trial, where we recorded the amount of time a test female swam in contact with the glass sides of the small tanks. We used three criteria for acceptance of a trial: (1) the test female had to be actively swimming, (2) she had to explore all parts of the large aquarium, and (3) she had to spend at least 3 min with both stimulus males. Trials were only started if both sets of test males developed breeding coloration and courted the female. The standard length (tip of snout to base of caudal peduncle) was measured with dial callipers on all fish used in these trials. Stimulus fish in each of the small aquaria were matched for size as much as possible. Since *C. maya* are larger than *C. labiosus* (often by a factor of two), we selected the smallest adult, reproductively active, *C. maya* males (\bar{x} = 47.8 mm, SE = 0.7 mm, range = 43–52 mm Standard Length) and the largest *C. labiosus* males (\bar{x} = 39.2 mm, SE = 0.6 mm, range = 35–46 mm SL) to minimize these size differences and to ensure that males would behave normally. Large *C. maya* males fail to develop breeding coloration if they are placed in small aquaria. To control for a side bias, we switched the position of the males and tested the female on the following day. Thus each female was tested twice. We randomized the presentation of the stimuli. All fish used in these experiments were adults with prior breeding experience.

We tested visual preferences of 16 *C. labiosus* females (\bar{x} = 36.2 mm, SE = 0.2 mm, range = 27–43 mm SL). One trial was discarded because the female spent less than 3 min viewing the males. Fifteen *C. maya* females were tested (\bar{x} = 38.1 mm, SE = 0.4 mm, range = 32–41 mm

SL). Of these, three were discarded either because they failed to explore the entire test aquarium or because they spent less than 3 min viewing the males.

CHEMICAL CUES

We used a 100-l experimental aquarium (100 cm × 35 cm × 35 cm) filled up to 80% with water. Two vertical lines drawn on the front of the aquarium visually divided it into three compartments of equal size. Two small aquaria (each 20 cm × 30 cm × 23 cm) containing 4 l of the test water were placed over this experimental tank. Silicone hoses fed the water from the two small aquaria 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. We adjusted the inflow valves of both hoses so that they delivered test water at a flow rate of 230 ± 2 ml per min. The two side walls of the experimental aquarium which were part of the two outer compartments into which the test water was delivered, were covered with tan-colored paper to eliminate the female's reflection when she followed the olfactory trials of the test water. While setting up these experiments we initially observed that females would be distracted by the reflection of their images and would spend disproportionate amounts of time examining their reflections rather than orienting toward the drip lines delivering water from the males. To eliminate responses to observers or other objects in the laboratory, we illuminated the tank with a fluorescent light strip (GE 'Plant and Aquarium' F20T12-PI/AQ) mounted directly over the tank but turned off the overhead lights in the laboratory. The test water was taken from 50-l aquaria with aeration systems containing either four *C. maya* or four *C. labiosus* males. Males remained in the aquaria for 24 h without being fed, to avoid food contamination of the trials. After each set of trials with a female the unused water from four males was discarded and a new set of four males were used to test preferences of the next female.

After every trial the small aquaria were replenished with test water from *C. maya* and *C. labiosus* males, the experimental apparatus was thoroughly cleaned with a clorox solution, and the experimental aquarium was refilled. The small delivery hoses were labelled to ensure that they would be used for test water of the same species throughout the experiment.

A test female was introduced into the experimental aquarium. As soon as the female remained in the middle of the compartment, we started the flow of the test water. The observation 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 aquarium. 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. In order to eliminate potential familiarity with odours of conspecifics, females and stimulus males came from different stock aquaria. Each female was only used for one trial which consisted of two presentations of the same set of stimuli. We measured olfactory preferences of 17 *C. labiosus* females and 15 *C. maya* females. Two *C. labiosus* and three *C. maya* trials were discarded because females failed to move between the two end compartments of the aquarium in either the first or the second presentation of male stimuli.

STATISTICAL ANALYSIS

Data were analysed with a non-parametric Wilcoxon-signed-ranks test since female responses to visual and chemical cues were not normally distributed. All significance levels were two tailed. Preference of an individual female was determined by calculating the proportion of time she spent with the conspecific male (time with conspecific male – time with heterospecific male/time with both males). A chi-square analysis was used to test for differences among females in the tendency to associate with conspecific and heterospecific males.

RESULTS

VISUAL CUES

C. maya females spent significantly more time with conspecific males ($z=3.09$, $P=0.002$, Fig. 1). All 12 females preferentially associated with conspecific males (Fig. 2). *C. labiosus* females did not visually discriminate between conspecific and heterospecific males ($z=1.56$, $P=0.12$, Fig. 1). Only 9 of 15 *C. labiosus* females showed a visual preference for their own males ($\chi^2=2.80$, $df=2$, $P>0.1$; Fig. 2).

CHEMICAL CUES

C. maya females spent significantly more time with conspecific males ($Z=3.02$, $P=0.003$, Fig. 3). Only one of the females was ambiguous in her choice of males and divided her time equally between the compartments with the test water of the conspecific and the heterospecific males ($\chi^2=15.4$, $P<0.005$, Fig. 4). *C. labiosus* females spent more time in the part of the tank with the test water of conspecific males ($Z=1.96$, $P=0.05$). The responses of individual females varied, although most clearly preferred the odour of their own males ($\chi^2=8.40$, $P<0.025$, $df=2$; Fig. 4).

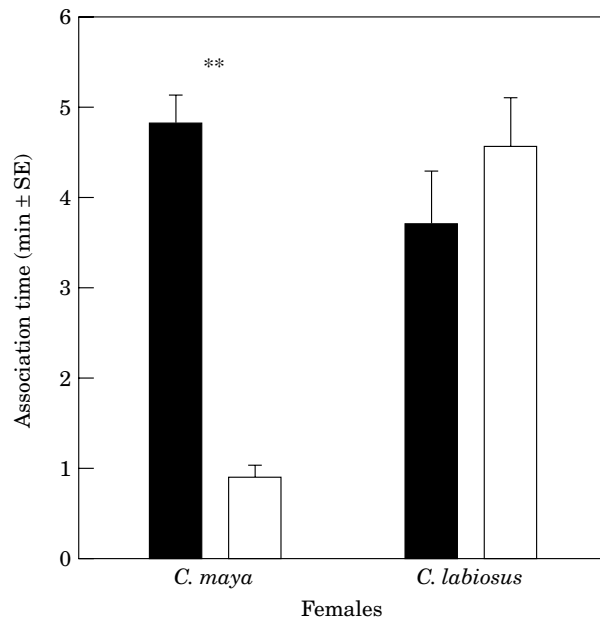


Figure 1. Time *C. maya* females ($N=12$) and *C. labiosus* females ($N=15$) spent with *C. maya* (■) and *C. labiosus* (□) males during visual trials. ** $P<0.001$. Only *C. maya* females visually preferred conspecific males.

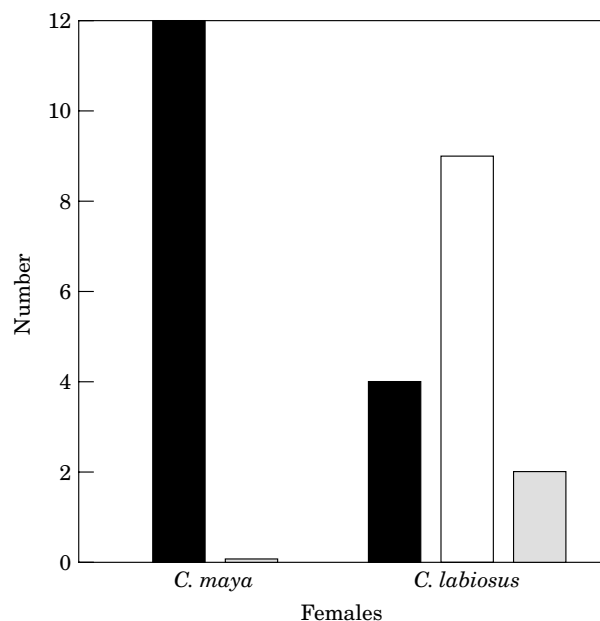


Figure 2. Number of *C. maya* and *C. labiosus* females with a visual preference for *C. maya* (■) and *C. labiosus* (□) or both (▣) males.

DISCUSSION

Visual recognition of conspecific males is asymmetrical; it is clearly present in *C. maya* females but not in *C.*

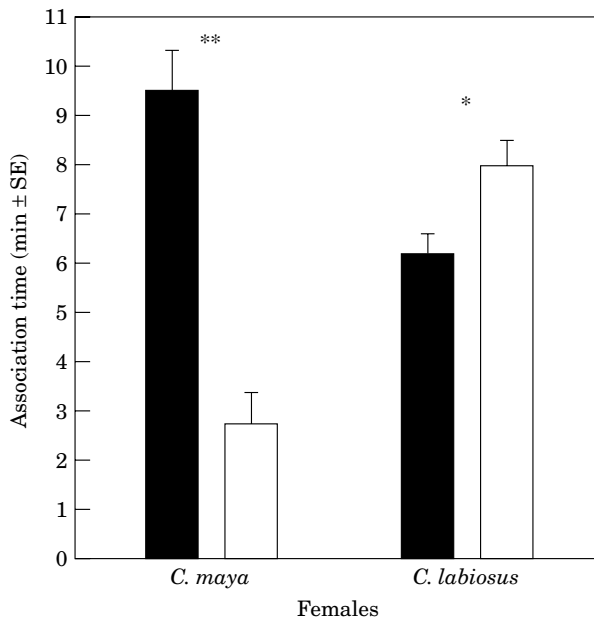


Figure 3. Time *C. maya* females ($N=12$) and *C. labiosus* females ($N=15$) spent with *C. maya* (■) and *C. labiosus* (□) males during chemical trials. * $P<0.05$, ** $P<0.001$. Females of both morphospecies preferred conspecific males.

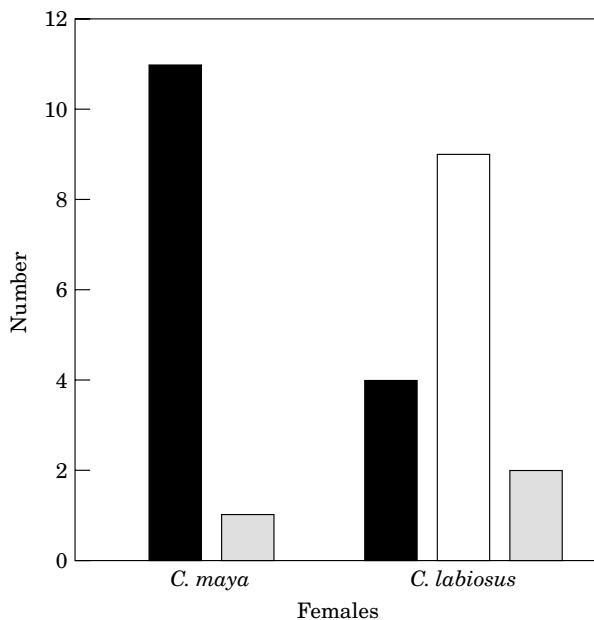


Figure 4. Number of *C. maya* and *C. labiosus* females with a chemical preference for *C. maya* (■) and *C. labiosus* (□) or both (▒) males.

labiosus females. The inconclusive results for the *C. labiosus* visual trials suggest that *C. labiosus* females lack the ability to discriminate between conspecific and *C. maya* males based on visual attributes, such as size and breeding coloration. It is possible that the variation in the responses of *C. labiosus* females to *C. maya* males may have been in part due to a preference for larger males overriding a preference for conspecific males. Females of many species of fish, including *C. variegatus*, a close congener of these pupfish, tend to prefer large males (pers. observ.; Kodric-Brown, 1990; Nagel & Schluter, 1998). Our results did not allow us to differentiate between these two hypotheses. There was no positive correlation between female size and the proportion of time females spent viewing *C. maya* males ($r_s=0.165$, $P=0.536$); thus larger females were no more likely to show a preference for *C. maya* males than smaller females.

Both *C. maya* and *C. labiosus* females associated with the odours of conspecific males, indicating that premating isolating mechanisms based on olfactory cues are present in both species; however, they seem to be stronger for *C. maya* than for *C. labiosus*. Since all fish were fed the same diet in the laboratory, differences in chemical cues between species presumably are genetically determined rather than environmental (Andersson, 1994; Bakker & Pomiankowski, 1995).

The *Cyprinodon* species flock has several unusual features which provide unique opportunities to study the evolution of mate-recognition systems in sympatry. This species flock is of recent (post-Pleistocene) origin, and its members show various stages of reproductive isolation (Strecker & Kodric-Brown, 1999, 2000). Asymmetries in mate recognition systems based on different sensory modalities suggest that selection acts, at least in this case, somewhat independently on each sensory modality. At least one species, *C. maya*, has diverged genetically as well as morphologically and behaviorally, and is reproductively isolated from two other species (*C. labiosus* and *C. beltrani*). *C. labiosus* is reproductively isolated from *C. maya* (as well as *C. beltrani*) based on chemical, but not visual cues, suggesting that discrimination among potential mates based on visual cues evolves more slowly than discrimination based on olfactory cues. *C. beltrani* is considered to be the most basal member of the species flock and is similar in morphology and diet to the sister species *C. artifrons* from the coastal lagoons of the Yucatan peninsula. It lacks the ability to discriminate between conspecific males and those of the other two species (*C. maya* and *C. labiosus*; Strecker, 1996; Strecker & Kodric-Brown, 1999). Thus, among the three members of the pupfish species flock examined to date (Fig. 5), premating isolating mechanisms range from none (*C. beltrani*), to incomplete, asymmetric,

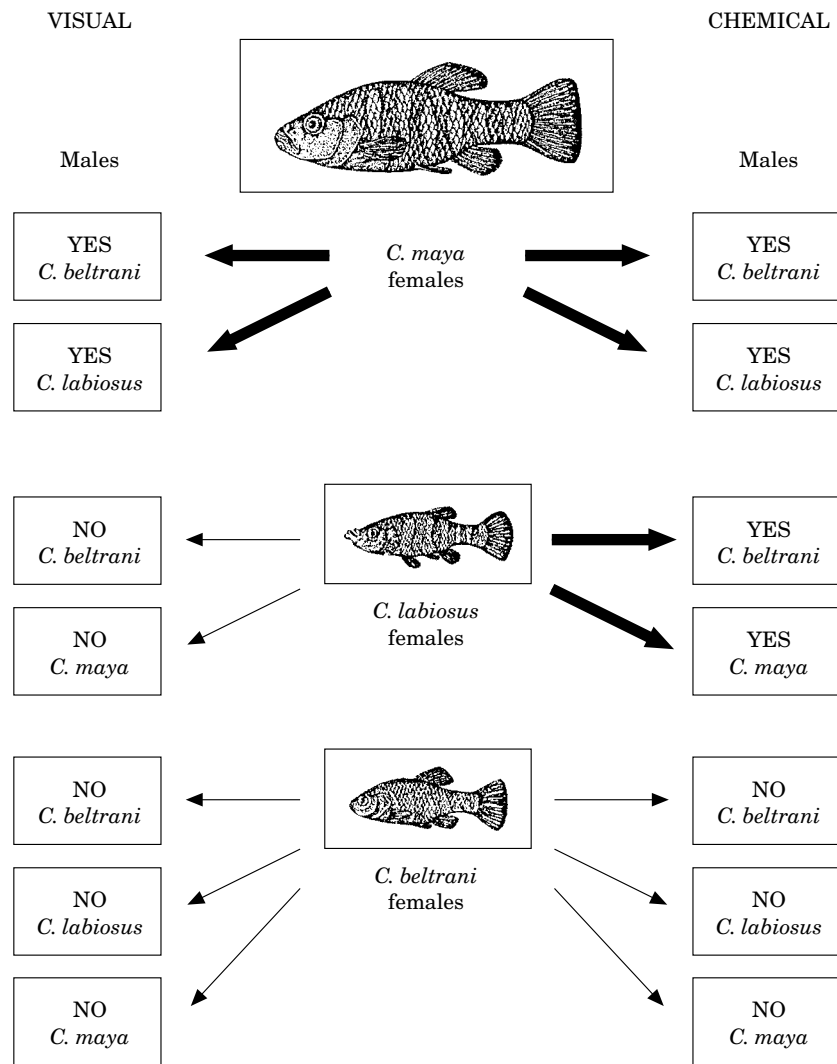


Figure 5. A summary of responses of *C. beltrani*, *C. labiosus*, and *C. maya*, females to visual and chemical cues of conspecific and heterospecific males.

and presumably still evolving (*C. labiosus*), to complete (*C. maya*).

Study of premating isolating mechanisms also provides insights into the various stages of the speciation process, from incipient (*C. labiosus* and *C. beltrani* interactions) to complete (behavioral interactions between *C. maya* with males of the other two species). In members of this species flock the strength of premating isolating mechanisms and the reliance on both olfactory and visual sensory modalities track the differentiation of morphological traits, such as head and mouth structures, that facilitate trophic differentiation. Selection for dietary differentiation seems to be the main force causing sympatric speciation. The observation that conspecific recognition is primarily based on chemical rather than visual cues, suggests

that trophic differentiation rather than runaway sexual selection seems to be the primary selective pressure promoting speciation in sympatry (Turner & Grosse, 1980; Schluter, 1996). However, sexual selection may play a role in reinforcing the isolation linked to trophic specialization (Andersson, 1994; Seehausen, 2000; Wilson, Noack-Kunmann & Meyer, 2000). An example may be the visual discrimination seen in *C. maya*.

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