

Divergent evolution of feeding substrate preferences in a phylogenetically young species flock of pupfish (*Cyprinodon* spp.)

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Abstract A fundamental question in sympatric speciation is how trophic divergence is achieved. We used an extremely young (<8,000 years) species flock of pupfish (*Cyprinodon* spp.) from Laguna Chichancanab in south-eastern Mexico to examine divergent evolution of preferences for different feeding substrates. In a test aquarium, we presented four feeding substrates (sand, gravel, a plastic plant, and blank bottom), but no actual food was offered. The four feeding substrates were chosen to mirror the most common substrate types in Laguna Chichancanab. Previous studies demonstrated that benthic food items prevail in the diet of most *Cyprinodon* species. *C. beltrani* preferred sand, whereas *C. labiosus* preferred gravel. F₁ hybrids of both species showed intermediate preferences. *C. maya* searched for food equally at all substrates. As the test fish were reared under identical laboratory conditions (i.e., in the absence of feeding substrates), the species-specific preferences appear to be genetically fixed, suggesting rapid divergent evolution of feeding behaviors.

Keywords Sympatric speciation · Trophic segregation · Niche partitioning · Foraging behavior · Habitat choice

Introduction

An increasing body of literature provides compelling evidence for sympatric diversification, which can ultimately result in sympatric speciation (e.g., Doebeli and Dieckmann 2000; Johannesson 2001; Coyne and Orr 2004; Savolainen et al. 2006). For example, ecotypes in the marine snail *Littorina saxatilis* appear to have evolved on multiple occasions in sympatry along an ecological gradient consisting of near-shore/offshore habitats (Johannesson et al. 1995; Cruz et al. 2004; Quesada et al. 2007). Fish species flocks are well suited to examine mechanisms of sympatric speciation (e.g., Sturmbauer and Meyer 1992; Schliewen et al. 1994; Barluenga et al. 2006; see also Schliewen et al. 2006). Species flocks are defined as groups of closely related species that descended from a common ancestor and inhabit a restricted area endemically (Mayr 1942; Greenwood 1984; Ribbink 1984).

Two major factors seem to play an important role in adaptive radiations, namely morphological, physiological, or behavioral differentiation that allow for the differential use of ecological resources (Rosenzweig 1995; Dieckmann and Doebeli 1999; Schluter 2000, 2001; Arnegard and Kondrashov 2004; Feulner et al. 2007) and assortative mating, leading to reproductive isolation (Seehausen et al. 1997; Nagel and Schluter 1998; Strecker and Kodric-Brown 1999; Coullidge and Alexander 2002; Cruz et al. 2004; McKinnon et al. 2004; Boughman et al. 2005; Hollander et al. 2005). Differences in food and/or habitat choice are thought to play a key role to facilitate niche partitioning in sympatric species (McKaye 1980; McPhail

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1992; Bouton et al. 1997; Rolán-Alvarez et al. 1997; Erlandsson et al. 1998; Carballo et al. 2005).

In the present study, we examined differences in feeding substrate preferences in a species flock of Mexican pupfishes (*Cyprinodon* spp.). In contrast to many highly diversified fish species flocks, the sympatric pupfishes from Laguna Chichancanab provide an example of a phylogenetically young species flock that comprises comparatively few species (Humphries and Miller 1981; Humphries 1984a, b; Strecker 2002, 2004, 2005). The sympatric *Cyprinodon* species are morphologically distinct (especially with regard to viscerocranial structures; Humphries and Miller 1981; Horstkotte 2005; Fig. 1) but show little overall genetic divergence based on neutral molecular markers (Humphries 1984b; Strecker et al. 1996; Strecker 2006a). An analysis of the geological history of this lake suggests that it desiccated approximately 8,000 years ago (Covich and Stuiver 1974). The observed pattern of weak genetic differentiation at neutral loci, with several shared alleles, and the fact that the derived phenotypes are not found outside Laguna Chichancanab suggest that the sympatric speciation of pupfishes likely occurred after that time (Strecker 1996, 2006a), and any differentiation among species—morphological and behavioural—must be viewed as a rapid evolutionary process (Strecker et al. 1996; Strecker 2004; Plath and Strecker 2008). Also, in other, more species-rich species flocks like the cichlid flock from Lake Victoria, pronounced morphological differentiation in the face of weak genetic

differentiation at neutral loci and in conjunction with a young geological age of the lake water body suggest rapid (explosive) intralacustric speciation (Meyer et al. 1990; Johnson et al. 1996; Nagl et al. 2000). Another phylogenetically young species flock of pupfishes comprising at least three divergent morphotypes is described from a lake on the Bahamas (San Salvador Island; Holtmeier 2001).

We examined differences in the preferred feeding substrate types among three species from Laguna Chichancanab. A gut content analysis revealed that the Laguna Chichancanab pupfishes primarily ingest benthic food items (Horstkotte and Strecker 2005). Therefore, we focused on different bottom substrate types and asked if the species would differ in feeding substrate preferences, pointing toward trophic niche segregation. By observing the response of naive, lab-reared focal fish to different feeding substrates, we could determine if differences in behavior among species are heritable.

Materials and methods

Study system

Laguna Chichancanab (Yucatán and Quintana Roo, Mexico) is characterized by extreme abiotic conditions with concentrations of calcium sulfate reaching saturation (Covich and Stuiver 1974; Strecker 1996; Hodell et al. 2001). Extreme

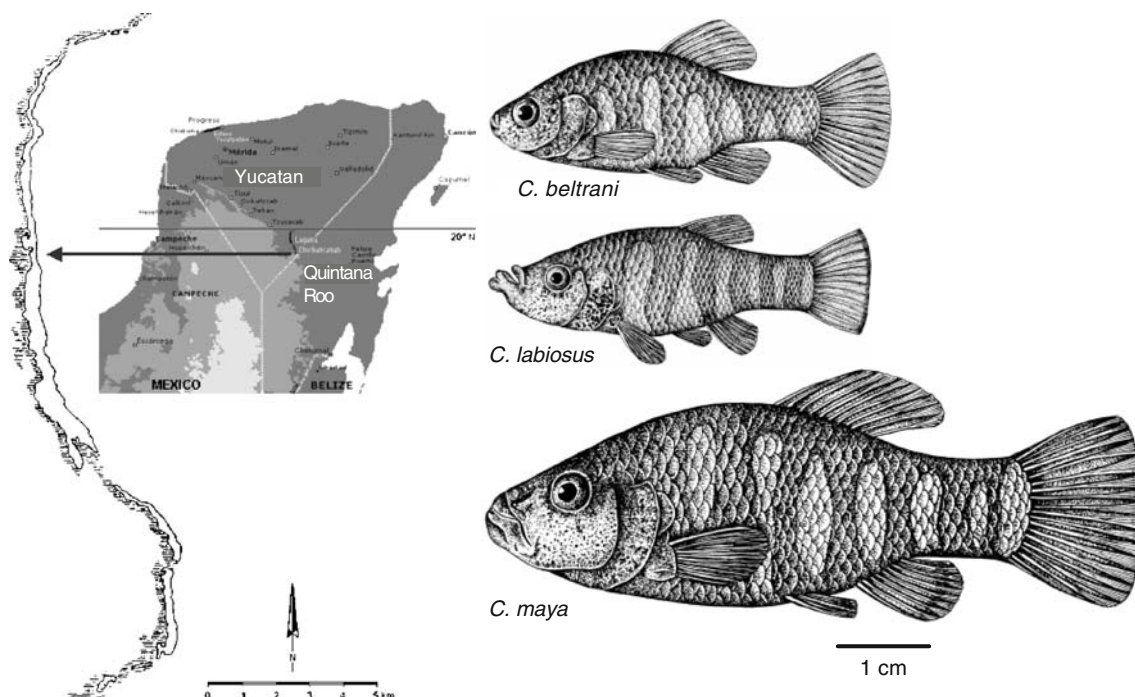


Fig. 1 *Left*, Laguna Chichancanab in Mexico (map from Microsoft Encarta). *Right*, the three sympatric *Cyprinodon* species examined in this study. Note the wider mouth and larger body size in *C. maya*,

reaching up to 80 mm standard length and fleshy lips in *C. labiosus* (see Humphries and Miller 1981)

aquatic habitats typically exhibit decreased species richness (e.g., Tobler et al. 2006; for Laguna Chichancanab see Strecker 1996, 2006b). Three species of algae of the genus *Chara* (*C. cianegaensis*, *C. haitensis*, and *C. hornemanni*) represent the only form of submersed vegetation in Laguna Chichancanab (Strecker 1996). Only very few fish species inhabit Laguna Chichancanab, namely the seven members of the *Cyprinodon* species flock (*C. beltrani*, *C. simus*, *C. labiosus*, *C. verecundus*, *C. esconditus*, *C. maya*, and *C. suavius*; Cyprinodontidae) and one species of mosquitofish (*Gambusia sexradiata*, Poeciliidae; Humphries and Miller 1981, Humphries 1984a; Strecker 2002, 2005). This relaxation of inter-specific competition by non-congeneric species is thought to have given rise to the radiation of pupfishes due to the availability of unoccupied niches (Strecker 1996, 2004; Strecker et al. 1996).

The Laguna Chichancanab pupfishes are genetically very similar to *C. artifrons*, a widespread species in coastal brackish waters of Yucatan, suggesting that *C. artifrons* is the sister species to the species flock (Humphries 1984b; Strecker et al. 1996; Strecker 2006a; Echelle et al. 2005). *C. beltrani*, the most common pupfish species in Laguna Chichancanab (60–85% abundance; Humphries and Miller 1981; Strecker 2006b), is morphologically similar to *C. artifrons* (Humphries and Miller 1981; Horstkotte and Strecker 2006). The other, less abundant species show characteristic morphological changes, such as a wider mouth and larger body size in *C. maya*, reaching up to 80 mm standard length (Humphries and Miller 1981), or fleshy lips in *C. labiosus* (Humphries and Miller 1981; Strecker 2005).

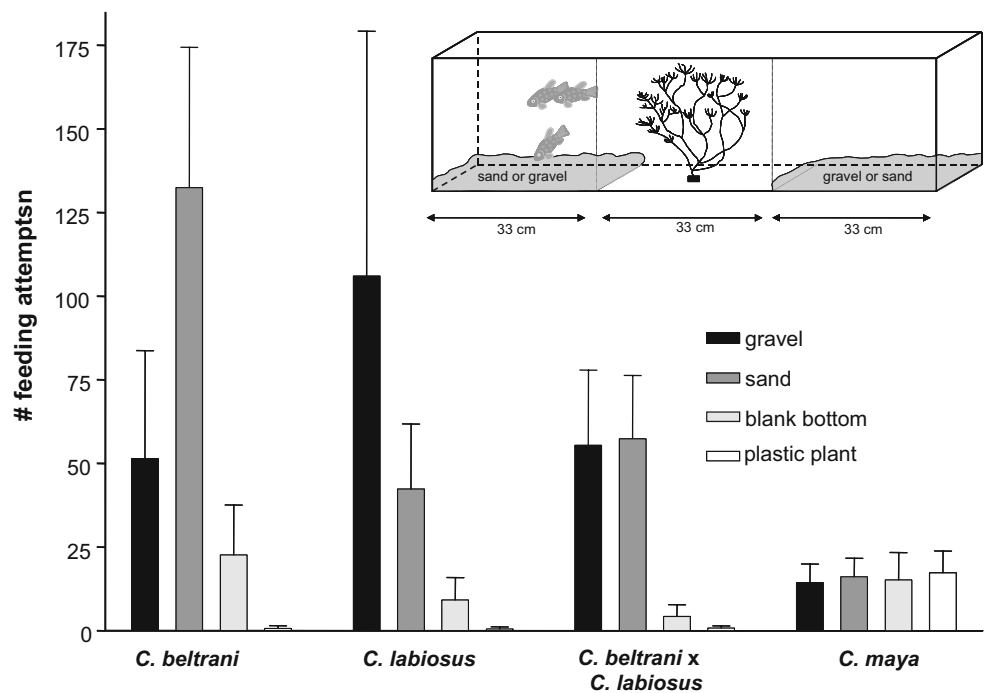
Study species and their maintenance

Test fish were offspring of wild-caught fish of the second to third laboratory generation. To avoid sexual and/or territorial interactions between the test fish, only females were used. We tested *C. beltrani* [mean (\pm SE) standard length, 31 \pm 1 mm], *C. labiosus* (33 \pm 2 mm), and *C. maya* (52 \pm 2 mm; Fig. 1). F₁ hybrids (*C. beltrani* females \times *C. labiosus* males; 32 \pm 2 mm) were included to further examine the heritability of feeding substrate preferences. Fish were maintained in 50–100 L aquaria at a temperature of 25°C and 5‰ salinity. The tanks did not contain any gravel, sand, or plants, such that the test fish were naive with respect to different feeding substrates prior to the tests. Test fish were fed exclusively with flake food and *Artemia* nauplii since hatching. All test fish were acclimated to the experimental room in blank 100 L aquaria, the dimensions of which were identical to the test tank, for at least 2 weeks before the tests and were not fed for 4 days before the tests.

Feeding experiments

A 100-L aquarium (100 \times 35 \times 35 cm) was visually divided into three equal compartments (Fig. 2). Three sides were covered by black plastic foil. Three 40-W incandescent bulbs, installed 25 cm above the test tank, provided light. Feeding substrates were chosen to mimic the major substrate types found in Laguna Chichancanab. The sympatric *Cyprinodon* species seem to exploit benthic food sources in nature (Horstkotte and Strecker 2005). Prior to the tests, bright gravel (grain diameter, 5–10 mm) and sand

Fig. 2 The mean (\pm SD) numbers of feedings attempts at the different substrate types (gravel, sand, the blank bottom, or the plastic plant) in *C. beltrani*, *C. labiosus*, the F₁ hybrids (*C. beltrani* \times *C. labiosus*), and *C. maya*. Inset figure: experimental setup (for details see main text)



(250–500 μm) were carefully washed and then sterilized overnight at 150°C. The two outer compartments of the test tank were equipped with a 3-cm layer of each sediment type (e.g., gravel left and sand right). We altered side assignments for each trial. The bottom of the middle compartment remained empty. Here, a *Chara*-like plastic plant as another potential feeding source was placed (Fig. 2). Hence, different feeding substrates, but no actual food source was offered. Initially, we did not expect the fish to feed on the blank bottom in the center. However, in preliminary tests some fish attempted to forage on the bottom also in this portion of the test tank, such that this “substrate type” was also considered in our present study.

To initiate a trial, three individually distinguishable females of a given species were introduced into the test tank. The female that started feeding first was considered the focal fish, and only its behavior was recorded. In previous tests, solitary females showed signs of distress (such as enhanced gill ventilation) and did not feed in this setup. After 30 min, for acclimatization, attempts to forage at the gravel, sand, plastic plant, and on the blank bottom were counted during a 30-min observation period. Foraging attempts are easily recognized as they involve opening of the mouth at the substrate. To account for potential side-biases, the females were placed back into the acclimatization tank after the first observation. Then, the gravel and sand were interchanged between the two outer compartments, and the measurement was repeated on the next day. Hence, each trial consisted of two test units. We summed the numbers of feeding attempts at each substrate type from the two test units. We tested ten individuals of each species and the hybrids. Each fish was tested in only one trial (consisting of two test units).

Statistical analyses

We tested for differences between the four groups in absolute numbers of feeding attempts (combined for all substrate types). Because the assumption of equal variance was violated even after applying standard transformation methods, a non-parametric Kruskal–Wallis test was employed. For post hoc pair-wise contrasts, Dunn’s test was used. To account for differences between species in the absolute number of feeding attempts, we used proportional data to test for differences in feeding preferences. The proportion of feeding attempts at each of the four feeding substrates [arcsine (square-root)-transformed] was the within-subjects factor (i.e., the repeated measurement), and ‘species/group’ was the between-subjects factor in a repeated-measures analysis of variance (ANOVA). Because the interaction term was significant in this analysis (see below), in a subsequent analysis, each group was analyzed separately while using one-way repeated measures ANOVA

on non-transformed data, and Tukey tests were used for post hoc contrasts.

Results

Absolute numbers of feeding attempts

There was a significant difference between the four groups in the combined number of foraging attempts ($H_3=21.19$, $P<0.001$; Fig. 2). A post hoc test showed that *C. maya* exhibited fewer foraging attempts than *C. beltrani* and *C. labiosus* ($P<0.005$ in both cases) but did not differ significantly from the hybrids ($P>0.05$).

Between-species comparison of preferred substrate types

The interaction term (substrate type \times group) had a significant effect ($F_{9,108}=35.52$, $P<0.0001$), indicating that the four groups of fish differed in their preferences (Fig. 2). Also the substrate type per se (i.e., the repeated measurement) had a significant effect ($F_{3,108}=147.01$, $P<0.0001$), indicating that gravel and sand were generally preferred over the other substrates. Species identity had no significant effect ($F_{3,36}=0.54$, $P=0.66$).

Within-species comparison of preferred feeding substrates

In *C. beltrani*, there was a significant difference in numbers of feeding attempts between substrate types ($F_{3,39}=61.76$, $P<0.001$; Fig. 2). The fish attempted to forage more on sand than on gravel, the plastic plant, or the blank bottom ($P<0.001$ in all cases) and also attempted to forage more on gravel than on the blank bottom ($P=0.046$) or the plant ($P<0.001$). Also in *C. labiosus*, a significant difference between substrate types was found ($F_{3,39}=17.97$, $P<0.001$). However, females of this species preferred gravel to sand ($P=0.003$) and the other substrate types ($P<0.001$ in both cases). In the hybrids, significant differences were also detected ($F_{3,39}=53.95$, $P<0.001$). Unlike the two parental species, the hybrids attempted to forage equally on sand and gravel ($P=0.99$) but preferred both substrates to the other substrate types ($P<0.001$ in all cases). *C. maya* females showed no preference at all ($F_{3,39}=0.34$, $P=0.80$).

Discussion

We examined the response of naive females of three sympatric pupfishes from Laguna Chichancanab to different feeding substrates. *C. beltrani* preferentially attempted to forage on sand, *C. labiosus* preferred gravel, while *C. maya* showed no

preference. Since lab-reared animals were employed and rearing conditions were identical for all species (i.e., all fish were reared in the absence of feeding substrates), the behavioral differences described here appear to be heritable. This interpretation is corroborated by the analysis of the F₁ hybrids (*C. beltrani* × *C. labiosus*), which showed intermediate expression of both parental preferences. Likewise, intermediate expression of shoaling and aggressive behavior was found in F₁ hybrids between *C. beltrani* and *C. simus* (Plath and Strecker 2008). Because the phylogenetic age of the Laguna Chichancanab species flock is estimated as less than 8,000 years (Strecker et al. 1996 for a discussion), our results suggest that rapid behavioral differentiation among the sympatric forms has occurred.

Differences in food and/or habitat choice are thought to play a key role for niche partitioning in sympatric species (e.g., Goldschmidt et al. 1990; Bouton et al. 1997; McKaye 1980; McPhail 1992), and indeed, the Laguna Chichancanab pupfishes differ in gut contents (Stevenson 1992; Horstkotte and Strecker 2005). While the very abundant *C. beltrani* almost exclusively feed on detritus (92.7% of dry weight) just like most *Cyprinodon* species outside Laguna Chichancanab (Soltz and Naiman 1978), the other sympatric species feed more on different benthic invertebrates (Horstkotte and Strecker 2005). Detritus constitutes only 55.2% of the diet in *C. labiosus*; 27.2% of their diet consists of amphipods (*Hyalella azteca*), and their diet is supplemented by small numbers of ostracodes, insect larvae, and small aquatic snails (*Pyrgophorus coronatus*). Finally, in *C. maya*, detritus makes up only 26.8% of their diet, followed by ostracodes (19.3%), mussels (*Lucina* sp.; 17.6%), snails (17.1%; including the larger *Physella cubensis*), fishes (8.0%; *Gambusia sexradiata* and smaller *Cyprinodon* spp.), and *Chara* spp. (7.9%). The difference between the detritivorous *C. beltrani* and the other, more carnivorous *Cyprinodon* species is also reflected in relative gut lengths: The gut of *C. beltrani* is, on average, twice as long as the guts of the other sympatric species (Horstkotte and Strecker 2005).

Our results show that this trophic segregation is not only achieved by morphological differentiation (Fig. 1), but also divergent evolution of preferences for feeding substrates seems to play an important role. The detritivorous *C. beltrani* showed a preference for the finest substrate also in our laboratory experiments. *C. labiosus* prey upon medium-sized (approximately 3 mm; J. H., personal observation) benthic amphipods, which probably hide between pebbles, and in cracks and crevices on rocky ground. Finally, *C. maya* uses a wide food spectrum, which was reflected by *C. maya* foraging equally on all substrate types in our experiments. *Chara* spp. may be ingested because invertebrates are attached to the algae. Interestingly, *C. maya* was the only species that also attempted to forage on the *Chara*-like plastic plant in our experiments. Unfortunately, no data

on feeding substrate preferences in the natural habitat are available. Hence, behavioral observations on the feeding behavior of the sympatric pupfishes from Laguna Chichancanab are highly warranted.

Morphological differentiation and behavioral differences in feeding substrate preferences are probably inextricably linked. For example, the longer snout of *C. labiosus* seems particularly suited for searching for invertebrates between pebbles. We propose a future experiment testing whether the viscerocranial morphology and feeding substrate preferences are indeed linked: By examining a reasonably large number of F₂-hybrids from crosses between *C. beltrani* and *C. labiosus*, it should be possible to test for a correlation between viscerocranial morphology and feeding substrate preferences. This experiment, however, was not feasible in the course of the present study.

In summary, rapid evolution of morphological structures and feeding substrate preferences may interact in facilitating niche partitioning and trophic segregation in sympatric speciation events.

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