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Behavioral diversification in a young species flock of pupfish (*Cyprionodon* spp.): shoaling and aggressive behavior

Martin Plath · Ulrike Strecker

Abstract Increasing empirical and theoretical evidence supports the idea that sympatric speciation is operating, for example, in species flocks comprising several closely related fish species within one lake. Divergent natural selection (promoting spatial and food niche partitioning) and sexual selection (assortative mating) have been identified as key selection factors in intralacustric adaptive radiations. However, the evolution of social behaviors accompanying such adaptive radiations is less well understood. Using a phylogenetically young species flock of pupfish (*Cyprinodon* spp.) as a model, we examined differences among six sympatric species and compared their shoaling, aggressive, and territorial behaviors with that of a sister species (*C. artifrons*). Despite an estimated age of the species flock of less than 8,000 years, pronounced behavioral differentiation was found. *C. simus*, the smallest species in the flock, shoaled more than the other species and was less aggressive and less territorial than *C. beltrani*. F$_1$-hybrids between *C. simus* males and *C. beltrani* females showed an intermediate expression of shoaling and aggressive behavior. Niche partitioning among the members of this species flock appears to be accompanied by rapid divergent evolution of social behaviors. We discuss the potential role of phenotypic plasticity and within-species variation of social behaviors for such rapid behavioral diversifications in sympatric speciation processes.

Keywords Adaptive radiation · Group living · Phenotypic plasticity · Sympatric speciation · Territoriality

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Introduction

Fish species flocks are of prime interest in evolutionary biology because they are particularly suited to examine mechanisms of speciation (e.g., Sturmbauer and Meyer 1992; Strecker et al. 1996; Seehausen et al. 1997; Barluenga et al. 2006; Strecker 2006a). Species flocks are defined as groups of closely related species that have descended from a common ancestor and inhabit a restricted area endemically (Mayr 1942, 1963; Greenwood 1984; Ribbink 1984). Some fish species flocks like those comprising the numerous cichlid species of the Great East African Lakes (Meyer et al. 1990; Sturmbauer and Meyer 1992), the diverse cyprinodontid fishes from Lake Titicaca in Peru (Lauzanne 1982, 1992; Parenti 1984; Villwock 1986), or the cyprinid species flock from Lake Lanao in the Philippines (Herre 1933; Kornfield and Carpenter 1984) served as model systems to study adaptive radiation and sympatric speciation (Maynard-Smith 1966). Indeed, intralacustric sympatric speciation may account for the vast numbers of closely related fish species occurring within

some lakes (e.g., Schliewen et al. 1994; Johnson et al. 1996; Meyer et al. 1990; Barluenga and Meyer 2004; Barluenga et al. 2006; Strecker 2006a) or river systems (Feulner et al. 2006, 2007).

Beside sexual selection, i.e., assortative mating (Seehausen et al. 1997; Higashi et al. 1999; Strecker and Kodric-Brown 1999, 2000; Kodric-Brown and Strecker 2001; Couldridge and Alexander 2002), models of adaptive radiation typically consider some kind of morphological and/or physiological differentiation that allow for the differential use of ecological resources (Greenwood 1981; Yamaoka 1991; Schluter 1996, 2000; Arnegard and Kondrashov 2004). Differences in food and/or habitat choice are thought to play a key role to facilitate niche partitioning in sympatric species (Boutron et al. 1997; McKaye 1980; McPhail 1984, 1992; Sandlund et al. 1992; Stevenson 1992; Horstkotte and Strecker 2005). For example, the concept of competitive speciation (Rosenzweig 1978) assumes that previously uninhabited habitats (e.g., newly formed lakes) will allow an invading species to exploit a variety of thus far unoccupied niches. Competitive exclusion will then favor individuals that are particularly well adapted to one of these niches (see Schluter 1996, 2000), leading to very fast speciation due to disruptive selection, i.e., selection against intermediate types (see also Diekmann and Doebeli 1999; Losos 2000; Strecker 2004).

In contrast to many highly diversified fish species flocks, the sympatric pupfishes from Laguna Chichancanab in Yucatan, Mexico, provide an example of a phylogenetically young species flock that comprises comparatively few species (Humphries and Miller 1981; Humphries 1984a, b; Strecker 2002, 2005). An analysis of the geological history of this lake suggests that Laguna Chichancanab desiccated approximately 8,000 years ago (Covich and Stuiver 1974; Hodell et al. 1995). Hence, the speciation process of pupfishes within Laguna Chichancanab likely occurred after that time (Strecker et al. 1996; Strecker 2006a), and any differentiation among the sympatric species—morphological and behavioral—must be viewed as a rapid evolutionary process. In the present study, we examined behavioral diversification among the sympatric Laguna Chichancanab pupfishes. We hypothesized that (spatial) niche partitioning (e.g., Seehausen and Bouton 1997) will be accompanied by general differences in the social organization among species, such as shoaling, territorial, and aggressive behaviors.

Pupfish males are often highly aggressive (e.g., Dwyer and Beuling 1991) and defend territories (Itzkowitz 1977, 1979; Kodric-Brown 1977, 1978, 1983; Raney et al. 1953; Barlow 1961; but see Kodric-Brown 1981 for flexibility in breeding behavior), and small shoals are temporarily formed (Raney et al. 1953; Barlow 1961; Echelle 1973). Pupfish males usually establish territories in the shallow parts along the shorelines of lakes and rivers (Kodric-

Brown 1977, 1978; Barlow 1961). It seems straightforward to assume an evolutionary scenario in which a highly aggressive and territorial form of *Cyprinodon* entered the hitherto uninhabited Laguna Chichancanab. While there will be strong competition among those individuals inhabiting the littoral zone, the pelagic zone will represent an unoccupied spatial niche. A likely scenario would be divergent selection in at least two directions: Some species will remain highly aggressive, defend territories, and will show little shoaling behavior. Others may abandon territoriality and dwell afar from the shallow shoreline, so as to inhabit the open water column. In those species, shoaling behavior should be favored, which provides major benefits in terms of protection from predators (Godin 1986; Magurran 1990; Krause and Ruxton 2002), especially in fishes dwelling in the open water column (Pitcher and Parrish 1993). In turn, aggressive behavior may be reduced in the shoaling, nonterritorial species because less aggressive fish form more cohesive shoals (e.g., guppy, *Poecilia reticulata*: Magurran and Seghers 1991). Indeed, observations of large shoals of *C. simus* in pelagic regions of Laguna Chichancanab have been reported (Humphries and Miller 1981; Stevenson 1992).

We quantified shoaling behavior among six species from Laguna Chichancanab and a sister species (experiment 1), then compared aggressive behavior in a subset of species namely, the species with strikingly high shoaling (*C. simus*) and the most abundant form in Laguna Chichancanab, *C. beltrani* (Humphries and Miller 1981; Horstkotte and Strecker 2005), which shows low shoaling (experiment 2). Subsequently, we examined the consequences of this behavioral differentiation, and we asked if the pronounced behavioral differences between *C. simus* and *C. beltrani* (i.e., reduced aggression in *C. simus*) would result in *C. simus* males being inferior in competition for breeding sites (experiment 3). Finally, we asked if there are differences between *C. beltrani* and *C. simus* in the formation of breeding territories even in the absence of interspecific competition (experiment 4).

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 aquatic habitats typically exhibit decreased species richness (e.g., Tobler et al. 2006; for Laguna Chichancanab, see Strecker 1996, 2006a, b), and 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. suavium*) and one species of mosquitofish (*Gambusia sexradiata*; Alvarez 1949; Humphries and Miller 1981, 1984a; Strecker 2002, 2005). This leads to a situation where interspecific competition by noncongeneric species is almost absent, which is thought to have given rise to the radiation of pupfishes due to the availability of unoccupied niches (Strecker 1996; Strecker et al. 1996). The sympatric *Cyprinodon* species are morphologically distinct (especially with regard to viscerocranial structures; Humphries and Miller 1981; Horstkotte and Strecker 2005) but show little overall genetic divergence (Humphries 1984b; Strecker et al. 1996; Strecker 2006a).

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 2005). 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).

C. simus exhibits the most pronounced morphological changes: The body is more elongated, eyes are relatively larger, and the body sides are more plain than in *C. beltrani*

(Humphries and Miller 1981; Horstkotte and Strecker 2005). *C. simus* is the smallest species, reaching barely more than 35 mm standard length. It shows the most derived viscerocranium, with a vertical lower jaw and more but smaller teeth (Stevenson 1992; Horstkotte and Strecker 2005). During the last years, *C. simus* could not be found in the field anymore (Strecker 2006b), and it seems that *C. simus* has become very rare, if not extinct, in nature due to the introduction of nonindigenous fish species (Schmitter-Soto and Caro 1997; Fusilier 2001; Strecker 2006b).

Fish maintenance

For the behavior tests, offspring of wild-caught fish of the second to third laboratory generation were used. We collected all species of pupfish from Laguna Chichancanab (Fig. 1). *C. suavium* was not included in the experiments because not enough individuals were available from our stocks. The sister species *C. artifrons* was collected from coastal waters of the Estero Yucalpetén, Yucatán. Large, randomly outbred stocks were maintained in mixed-sex, single-species stock tanks of 100–200 l under a 16:8-h light/dark cycle. In the stock tanks and throughout the behavior tests, the temperature was adjusted at 25–27°C, and salinity was at 5‰. Each tank was equipped with an aerated gravel filter (10 cm height, 25 cm diameter). Fishes were fed twice a day with ad libitum amounts of Tetramin dry food, *Artemia* naupliae, live cladocerans, and *Tubifex* worms. Comparing the behavior of fishes that had been reared under identical conditions allows examining genetic differences. In addition, we included F_1 hybrids between *C. simus* males and *C. beltrani* females to further demonstrate the genetic basis of the examined behavior patterns.

Fig. 1 The six species of *Cyprinodon* from Laguna Chichancanab examined in this study. Note the pronounced morphological differentiation especially in viscerocranial structures (such as fleshy lips in *C. labiosus* or a vertical lower jaw in *C. simus*) pointing toward trophic niche segregation

C. beltrani

C. verecundus

C. labiosus

C. esconditus

C. maya

C. simus

1 cm
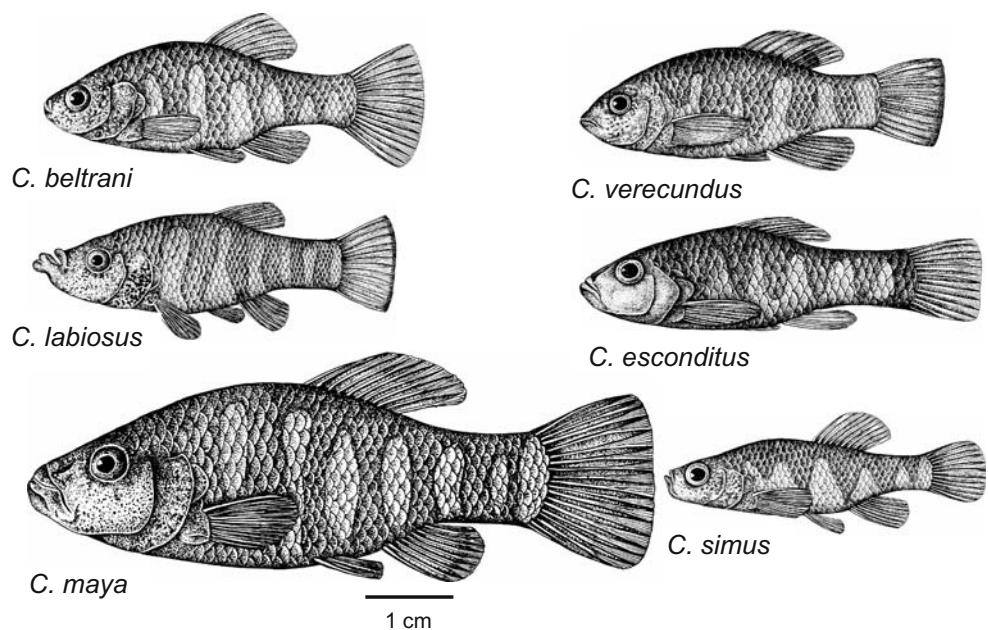
Experiment 1: shoaling

To determine shoaling tendencies, a large test tank (220× 57×50 cm length×width×height) was employed, which was filled with water to 34 cm height. An Osram® 40-W neon tube illuminated the tank from centrally above. UV light was absent. Two vertical lines drawn on the front visually divided a middle compartment (44 cm) from two outer compartments (each 88 cm). All tanks used for the behavior tests were covered with dark foil at three sides.

We placed a transparent Plexiglas cylinder (19.5 cm in diameter) in the center of the middle compartment to hold the stimulus shoal. Four stimulus fish were introduced into the cylinder and given 30 min for habituation. Then, a focal fish was gently introduced into one of the end sections of the tank and was given another 20 min for habituation. The side of introduction (left or right) was balanced. We measured the time the focal fish spent in proximity to the stimulus shoal (i.e., within the central compartment) during a 10-min observation period. The observer was sitting quietly approximately 2 m from the tank and was largely hidden behind a board in the middle of the room. We also quantified the time until the focal fish resumed swimming ("latency time"), i.e., the time the test fish spent motionless on the bottom after being introduced into the test tank (at the beginning of the 20-min acclimatization period). We considered the latency time until the focal fish resumed swimming (i.e., started exploring the novel environment) one potential indicator of boldness (short latency) vs. shyness (long latency time; e.g., Wilson et al. 1994).

To avoid an effect of familiarity on shoaling behavior (e.g., Griffiths and Maggurran 1997), the focal and stimulus fish were taken from different stock tanks. To avoid sexual attraction (see Lindström and Ranta 1993), tests were carried out with focal and stimulus fish of the same sex. We tested 15 individuals per sex of each of the six species from the Laguna Chichancanab, the F_1 hybrids (C. simus× C. beltrani) and the sister species C. artifrons. All focal fish were tested only once; however, due to the limited number of fish in our stocks, focal fish may have served as a stimulus fish in a subsequent trial but never on the same day. After a trial, all fish were measured for standard length to the closest millimeter (Table 1).

We used two-way analysis of variance (ANOVA) to analyze the data (time spent shoaling and latency time, respectively), whereby species identity was the between-subjects factor and sex was included as the within-subjects factor. To meet the assumptions of a normal distribution and equal variance, latency times were transformed as follows: $y'=\log[(y+0.5)\times10]$. For post-hoc contrasts, Fisher's protected least significant difference (PLSD) was employed.

Experiment 2: aggressive behavior

We tested whether species with a higher shoaling tendency would be less aggressive (e.g., Magurran and Seghers 1991 for Trinidadian guppies, Poecilia reticulata). Tests on aggressive behavior were conducted with a subset of species namely, C. simus and C. beltrani, the F_1 hybrids (C. simus×C. beltrani), and C. artifrons. C. artifrons and C. beltrani are territorial, so we predicted that they would shoal less but show more aggressive behavior. Another prediction for this experiment was that males in the territorial species would be more aggressive than females, since males but not females are territorial.

We used a 50-l aquarium (50×50×20 cm), the bottom of which was covered with bright multicolored gravel. The water was aerated by an air pump. To initiate a trial, four size-matched specimens of the same sex and species were introduced into the tank (between 09:00 and 10:00 hours). After 30 min for habituation, the number of aggressive behaviors among the four fish (biting, biting attempts, chasing, circling, and lateral displays; see Barlow 1961; Raney et al. 1953) was determined during a 30-min observation period. Measurement was repeated after 24 h, and data from both parts of a trial were summed. We tested $n=12$ groups of C. artifrons and $n=14$ groups of the other species and the hybrids. Half of the trials involved groups of males, and the other half involved females. All individuals were tested only once and were measured for standard length

Table 1 Mean (±SD) standard lengths (mm) of the focal (left) and stimulus fish (right) in the experiment on shoaling behavior (experiment 1)

	Males		Females	
	Focal fish	Stimulus fish	Focal fish	Stimulus fish
C. beltrani	33.4±5.2	33.6±4.0	29.8±3.6	26.1±3.5
F1 C. simus×C. beltrani	26.9±3.0	26.8±3.3	28.9±3.8	30.3±4.0
C. simus	27.0±2.2	26.8±2.3	25.2±2.7	24.5±1.9
C. labiosus	33.9±5.4	33.4±5.5	34.0±6.1	33.4±6.0
C. verecundus	37.5±5.1	37.0±5.1	35.3±6.7	35.3±6.7
C. esconditus	41.2±2.5	41.2±2.5	37.4±5.4	36.2±6.4
C. maya	48.3±3.2	47.9±3.5	45.0±3.5	45.8±2.7
C. artifrons	29.4±2.0	29.1±1.8	27.9±1.8	28.0±1.8

after the trials (mean±SD, *C. artifrons*, males=29.3± 1.9 mm, females=28.0±1.8 mm; *C. beltrani*, males= 33.5±4.6 mm, females=27.7±3.9 mm; *C. simus*, males= 26.9±2.2 mm, females=24.8±2.3 mm; F_1, males=26.8± 3.1 mm, females=29.6±3.9 mm).

Data on aggressive behavior (sum of aggressive behaviors of the four focal fish per trial) were analyzed using a two-way ANOVA on square-root-transformed data, in which species identity was a between-subjects factor and sex was the within-subjects factor. For post-hoc contrasts, Fisher's PLSD was employed.

Experiment 3: competition between *C. beltrani* and *C. simus* males for breeding territories

We determined if *C. simus* males are inferior competitors and less able to defend breeding territories because they are less aggressive compared with *C. beltrani* males. Previous experiments have shown that both species prefer to spawn on gravel (Strecker 1996), and in our stock tanks, all *Cyprinodon* species use gravel filters for spawning.

We used a 50-l test aquarium (see experiment 2) in which a gravel filter was placed centrally. To initiate a trial, one *C. beltrani* male (standard length=27.7±0.5 mm) and an equally sized *C. simus* male (27.8±0.6 mm) as well as one mature female of each species were introduced into the test tank and given 24 h for acclimatization. In the morning of the second day, we recorded which male defended a territory on the surface of the gravel filter. We considered a male defending a territory if the male chased the other male away from the filter. Territorial males also exhibited "patrolling" behavior near the surface of the gravel (Barlow 1961). We noted which male was defending the filter during three consecutive observations. Each observation lasted until territorial behavior was observed and was followed by a 30-min break. For the statistical analysis, ranks were assigned in a way that a male would yield a rank of +1 each time it was observed defending the filter. Rank sums from the three observations were compared between the two species using nonparametric Wilcoxon signed-rank tests. We conducted *n*=17 replicates.

Experiment 4: formation of breeding territories in *C. beltrani* and *C. simus*

We examined the formation of breeding territories in *C. beltrani* and *C. simus* in the absence of interspecific competition. We assumed that just like other pupfish occurring outside the Laguna Chichancanab (Raney et al. 1953; Barlow 1961; Itzkowitz 1977, 1979; Kodric-Brown 1977, 1978), *C. beltrani* males would defend breeding territories for extended periods of time, while males of the shoaling *C. simus* were predicted to form short-term breeding territories only sporadically. A large aquarium (220×50×57 cm) was visually divided into five equal compartments by four vertical lines drawn on the front (Fig. 2). A Petri dish (15 cm in diameter), filled with bright gravel, served as a territory and was placed in the center of each compartment. We placed a plastic plant as well as a stone (approximately 15 cm in diameter) between each of the five compartments to minimize visual contact between the compartments (Fig. 2). A neon tube above the tank provided a 16:8-h light/dark cycle.

To initiate a trial, five males and five females of a given species were introduced in the morning and could acclimatize for 3 days. The test fish were fed daily in the morning. Measurement started in the afternoon of the third day. We used a behavioral scan sampling method: In the course of 1 h, the behavior of the test fish was noted every 5 min. We noted how many males exhibited territorial behavior [i.e., patrolling on top of a Petri dish (Barlow 1961) or defending the Petri dish against intruders], and we noted how long (in terms of numbers of subsequent scan sampling intervals) individual males defended their territories.

We conducted another, independent analysis of shoaling behavior during this experiment. This allowed us to determine if differences among species in shoaling are evident also when spawning sites are present. Therefore, we determined the number of fish in each compartment during each scan, and we calculated the average number of compartments over which the test fish were distributed, as well as the maximum number of fish per compartment. We conducted seven trials with *C. beltrani* and eight trials with

Fig. 2 Experimental set-up for the experiment on the formation of breeding territories (experiment 4). For details, see main text. For display purposes, fish are depicted at a supernatural size (males: *dark gray*, females: *light gray*)
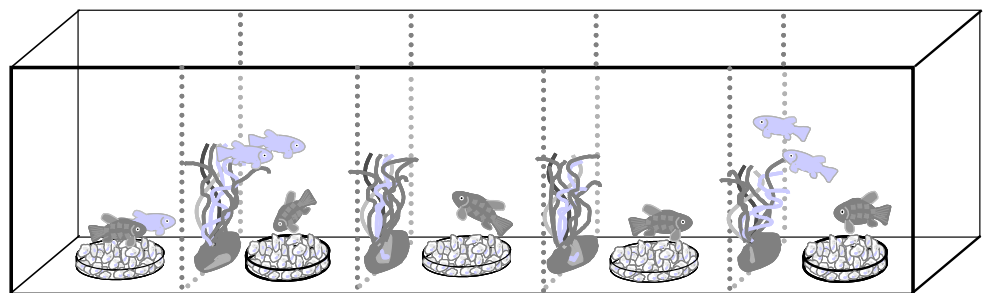
C. simus. All fish were only used in only one trial and were measured for standard length after the trials (*C. beltrani*, males=31.6 ± 4.8 mm, females=27.9 ± 3.9 mm; *C. simus*, males=27.2 ± 2.2 mm, females=24.8 ± 2.3 mm). For the statistical analysis, mean values for the 13 scan sampling points per trial were calculated. Because the data deviated from the assumptions of equal variance and normal distribution even after applying standard transformation methods, nonparametric tests (Mann–Whitney U tests) were employed for the statistical comparisons between species.

Results

Experiment 1: shoaling

Time spent shoaling

C. simus and *C. artifrons* spent considerably more time near the stimulus shoal than the other species examined (Fig. 3). Indeed, there was a statistically significant difference among species (ANOVA: $F_{7,\ 231}=7.91$, $P<0.0001$), and a post-hoc test (Fisher's PLSD) revealed that *C. simus* differed from the other species from the Laguna Chichancanab species flock ($P<0.05$) but did not differ from *C. artifrons* ($P=0.92$). Likewise, the shoaling tendency of *C. artifrons* was higher than that of all Laguna Chichancanab species except *C. simus* ($P<0.05$). There was a significant difference between the sexes in the time spent shoaling ($F_{1,\ 231}=9.13$, $P=0.0028$); overall, males spent more time shoaling than females (Fig. 3), suggesting that the more brightly colored males were more conspicuous stimuli. The interaction term "species"×"sex" had no significant effect ($F_{7,\ 224}=1.59$, $P=0.14$).
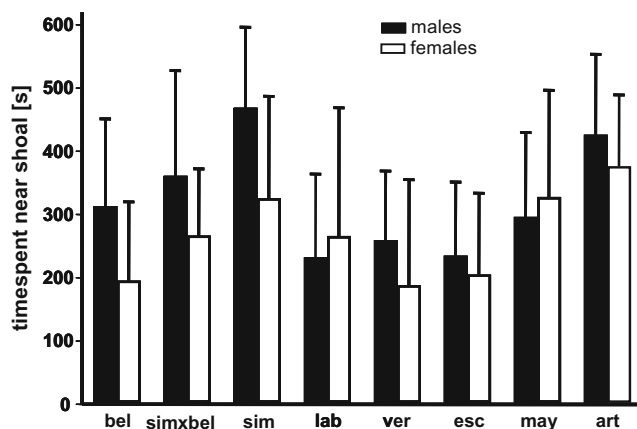
Fig. 3 The mean (±SD) time individual pupfish (*Cyprinodon* spp.) spent associating with a stimulus shoal consisting of four same-sex conspecifics (experiment 1). Species flock from the Laguna Chichancanab (from *left* to *right*): *bel Cyprindon beltrani*, *sim C. simus*, *lab C. labiosus*, *ver C. verecundus*, *esc C. esconditus*, *may C. maya*. Sister species from Estero Yucalpetén: *art C. artifrons*

Latency time

We found a significant difference among species in the time the focal fish spent motionless on the bottom when introduced into the test tank until they resumed swimming (ANOVA: $F_{7,\ 231}=7.10$, $P<0.0001$). *C. simus* exhibited longer latency times than all other species examined (Table 2; Fisher's PLSD: $P<0.01$ in all cases). All other post-hoc comparisons were nonsignificant ($P>0.05$). There was no significant difference between the sexes ($F_{1,\ 231}=0.25$, $P=0.51$). The interaction term "species"×"sex" was not significant ($F_{7,\ 224}=0.84$, $P=0.17$).
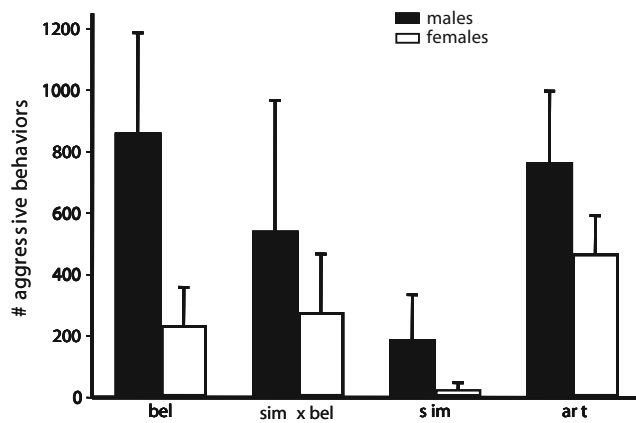
Experiment 2: aggressive behavior

C. artifrons, *C. beltrani*, *C. simus*, and the F_1 hybrids differed significantly in the number of aggressive behaviors (ANOVA: $F_{3,\ 51}=20.50$, $P<0.0001$; Fig. 4). *C. simus* exhibited significantly less aggressive behavior than *C. beltrani*, *C. artifrons*, and the F_1 hybrids (Fisher's PLSD: $P<0.0001$), and the F_1 hybrids were less aggressive than *C. artifrons* ($P=0.008$). All other pairwise comparisons were not significant ($P>0.05$). Generally, males were considerably more aggressive than females ($F_{1,\ 51}=30.08$, $P<0.0001$; Fig. 4). The interaction term "species"×"sex" was not statistically significant ($F_{3,\ 48}=1.66$, $P=0.19$).

Experiment 3: competition between *C. beltrani* and *C. simus*

In this experiment, one *C. beltrani* male and one *C. simus* male competed for access to a spawning site (a gravel filter). In only 4 of the 17 trials, a *C. simus* male defended the filter for one (three males) or two (one male) observation periods. Overall, the median score was significantly higher in *C. beltrani* males (3.00, interquartile range [IQR]=1.00) than in *C. simus* males (0.00, IQR=0.63; Wilcoxon signed-rank test: $z=-3.53$, $P=0.0004$, $n=17$).

Table 2 Mean (±SD) latency times until the focal fish started to swim in experiment 1

	Latency time (s)
C. beltrani	2.3 ± 5.3
F1 *C. simus*×*C. beltrani*	29.1 ± 34.3
C. simus	62.8 ± 92.7
C. labiosus	14.9 ± 34.3
C. verecundus	9.5 ± 23.6
C. esconditus	14.0 ± 21.7
C. maya	1.4 ± 4.8
C. artifrons	10.6 ± 16.0

Fig. 4 The mean (±SD) number of aggressive behaviors (per one hour observation time) in groups of four pupfish (experiment 2). For abbreviations, see Fig. 3

Experiment 4: breeding territories in *C. beltrani* and *C. simus*

Territorial behavior

On average (median), 2.23 *C. beltrani* males (IQR=1.44; 45% of the males) exhibited territorial behavior, but only 0.58 *C. simus* males (IQR=0.31; 12%) were observed defending territories. This difference was statistically significant (Mann–Whitney U test: $T=84$, $P<0.001$, $n_1=7$, $n_2=8$). No male was observed defending more than one Petri dish at a time. The two species differed significantly in the time individual males spent defending breeding territories (*C. beltrani*=11.25 min [IQR=9.85 min]; *C. simus*= 6.25 min [IQR=2.25 min]; Mann–Whitney U test: $T=80$, $P=0.004$, $n_1=7$, $n_2=8$). In *C. beltrani*, about half of the males defended their territories for only one observation period (5 min), but other territories were defended considerably longer, in some cases until the end of the observation period after 1 h. In *C. simus*, about three quarters of the territories were defended for only one observation period, and the longest period during which an individual male was observed defending a territory was five scan sampling intervals (20 min).

In all trials, females frequently approached males on the patches of gravel, and spawning was frequently observed. However, spawning was not quantified.

Shoaling

We also analyzed shoaling behavior in this experiment. The maximum number of fish per compartment was significantly higher in *C. simus* (6.39, IQR=2.15) than in *C. beltrani* (4.31, IQR=0.71; Mann–Whitney U test: $T=31$, $P=0.002$, $n_1=7$, $n_2=8$). Furthermore, *C. beltrani* were distributed over more compartments (4.23, IQR=0.50) than *C. simus*

(2.31, IQR=0.96; Mann–Whitney U test: $T=81.5$, $P=0.001$, $n_1=7$, $n_2=8$).

Discussion

Shoaling and aggressive behavior

In a between-species comparison of the pupfishes from Laguna Chichancanab, *C. simus* exhibited the strongest tendency to form shoals (experiment 1). In experiment 2, *C. simus* showed considerably less aggressive behavior compared with *C. beltrani* or *C. artifrons*. Because lab-reared animals were employed and rearing conditions were identical for all species, these behavioral differences likely have a strong genetic component. Shoaling as well as aggressive behavior of the F_1-hybrids (*C. simus*×*C. beltrani*) was intermediate in intensity/frequency to that of the parental species, suggesting that the observed differences among species are indeed genetic. Beside morphological differences (Humphries and Miller 1981; Horstkotte and Strecker 2005), behavioral differences are evident in the Laguna Chichancanab pupfishes despite little overall genetic differentiation (see Humphries 1984b; Strecker 2006a). The Laguna Chichancanab species flock is considered evolutionarily young (Strecker et al. 1996; Strecker 2006a); hence, the observed behavioral changes represent a rapid evolutionary event.

In other pupfish species occurring outside the Laguna Chichancanab, males are typically territorial and highly aggressive during the breeding season and, thus, do not shoal (*C. macularius*: Barlow 1961; *C. pecosensis*: Kodric-Brown 1977, 1978, 1983), even though females of such species may temporarily form small shoals (*C. variegatus*: Raney et al. 1953; *C. macularius*: Barlow 1961; *C. rubrofluviatilis*: Echelle 1973). However, because shoaling provides major benefits in terms of protection from predators (Seghers 1974; Godin 1986; Magurran 1990; Magurran et al. 1992; Krause and Ruxton 2002), even males of highly territorial pupfish species can be observed shoaling while they are not defending a territory (i.e., in species from temperate latitudes during winter: Barlow 1961; Echelle 1973).

Latency to approach the stimulus shoal as an indicator of boldness?

Long latency times in *C. simus* in experiment 1 are probably indicative of decreased boldness in this species. "Freezing" is a typical fear response of fishes in a novel environment (e.g., Brown and Smith 1996; Templeton and Shriner 2004). The adaptive significance of higher boldness in territorial species like *C. beltrani* or *C. artifrons* may be linked to the fact that those species must re-enter their

territories soon after disturbance, for example, after predator attacks. This, however, is not the case in the less territorial, shoaling *C. simus*. Population differences in behavioral traits associated with boldness have been found, for example, in sticklebacks (*Gasterosteus aculeatus*; Bell and Stamps 2004), perch (*Perca fluviatilis*, Magnhagen 2006), and Panamanian bishop (*Brachyraphis episcopi*; Brown et al. 2005). In our study, differences in latency times were found among different *Cyprinodon* species within the same lake. Because all fish were reared under standardized conditions and in the absence of predators, behavioral variation in this trait must be largely due to genetic differences among species (see also van Oers et al. 2004 for a genetic basis of boldness). Brown et al. (2007) described genetic variation in behavioral traits associated with boldness also for different populations of *B. episcopi* (Poeciliidae).

Territoriality

C. simus males were inferior to *C. beltrani* males when competing for access to breeding sites (experiment 3), suggesting that *C. simus* males use different microhabitats for spawning in nature (see McKaye 1980, for *Amphilophus citrinellum*). However, even when no *C. beltrani* males were present and all males had an opportunity to form a territory (experiment 4), the *C. simus* males "patrolled" breeding sites only for short periods of time, and the tendency to form shoals was strong also in this experimental situation. Along with the strong reduction of aggressive behavior (experiment 2), this suggests that *C. simus* males have adopted a different breeding strategy, whereby short-term breeding territories are formed while mixed-sex shoals approach spawning sites. It remains to be examined whether *C. simus* males form these short-term territories in deeper parts of the lake, where no *C. beltrani* males compete for territories.

It seems that differences in mating systems between the Laguna Chichancanab pupfishes are also reflected by differences in the expression of secondary sexual characteristics: Although a thorough investigation of color differences between the species of the flock remains to be carried out, it seems that sexually dimorphic ornaments are less developed in *C. simus*. For example, *C. beltrani* males are often almost entirely black, with a yellow margin at the caudal fin. Such conspicuous coloration is barely seen in *C. simus*. A likely explanation for a reduction in sexually dimorphic traits would be that intrasexual selection (aggressive competition among males for access to breeding sites) is reduced in *C. simus*. *C. simus* is the smallest species in the flock. Again, it seems likely that intrasexual selection selects for competitive abilities (i.e., large body size) in the territorial species but not in the shoaling *C. simus*. The role of female choice on male coloration and body size remains to be studied in this system.

Phenotypic plasticity and behavioral diversification

In other pupfishes, mating systems are known to be plastic, and mating systems depend on environmental constraints (Kodric-Brown 1981 for a review). Territoriality is found most frequently and is often accompanied by satellite and sneaky mating tactics (Kodric-Brown 1977, 1986). Where the spawning substrate is limited, also a dominance system can be found (Itzkowitz 1977; Kodric-Brown 1988), and at very low population densities, the formation of "consort pairs" can be seen (Kodric-Brown 1981). Where environmental conditions do not favor the formation of territories, "group spawning" is observed (Kodric-Brown 1981). We propose that phenotypic plasticity in the form colonizing Laguna Chichancanab was a source for the behavioral diversification within Laguna Chichancanab, with *C. beltrani* adopting the territorial strategy but *C. simus* adopting a strategy that resembles more the described "group spawning" (Kodric-Brown 1981).

Generally, adaptive radiations may occur when an invading species starts exploiting different, thus far unoccupied niches (e.g., Rosenzweig 1978). Divergent selection will then favor those phenotypes that are particularly well adapted to a given niche (Doebeli 1996; Diekmann and Doebeli 1999; Kondrashov and Kondrashov 1999). This scenario requires some variability in the colonizing form, which could be due to genetic variation or phenotypic plasticity. The results from this study suggest that behavioral plasticity may indeed play an important role. Our results underscore the proposed important role phenotypic plasticity plays in facilitating macroevolutionary processes (Pigliucci 2001; West-Eberhard 2003). Pigliucci (2005), for example, suggested that former phenotypic plasticity might decrease in a species if new environmental conditions persist. This may also be the case for species exploiting different niches within the same lake. Future studies will need to examine whether the Laguna Chichancanab pupfishes have indeed reduced behavioral plasticity as compared to other pupfishes like *C. artifrons*.

Sympatric speciation in the Laguna Chichancanab species flock

Altogether, the behavioral differentiations described here might act together with sexual selection by female choice (i.e., assortative mating) and disruptive natural selection (leading to trophic segregation) in the speciation process of the Laguna Chichancanab species flock (Horstkotte and Strecker 2005; Strecker 2004). Using gut content analysis, Horstkotte and Strecker (2005) demonstrated that differences in viscerocranial morphologies are reflected by different food preferences: For example, bivalves constitute a substantial proportion of the diet only in *C. verecundus*,

and only (large) *C. maya* consume small fish. While Horstkotte and Strecker (2005) found exclusively detritus in the guts of *C. simus*, Humphries and Miller (1981) reported on *C. simus* feeding on pelagic plankton. Indeed, it seems likely that the shoaling *C. simus* feed primarily on planctic organisms in the open water column. By contrast, in the highly territorial *C. beltrani*, males need to rely on detritus as a food base (see Horstkotte and Strecker 2005), which is the only food source they regularly encounter within their territories.

When comparing the ability to discriminate between conspecific and heterospecific males between *C. beltrani*, *C. labiosus*, and *C. maya* females, it was found that females of one species (*C. maya*) use both visual and chemical cues for mate choice (Strecker and Kodric-Brown 1999, 2000; Kodric-Brown and Strecker 2001). By contrast, *C. labiosus* females discriminated only when visual cues were available but not when chemical cues were provided. Finally, the most common form, *C. beltrani*, did not discriminate between conspecific and heterospecific males in either experiment. This may reflect a frequency-dependent component in mate choice behavior, with the least abundant species (*C. maya*) exhibiting the strongest mating preferences. Whether *C. simus* females also show such mating preferences for conspecific males remains to be studied. The present study suggests that shoaling behavior could act as another behavioral mechanism that leads to reproductive isolation between the sympatric species: *C. simus* form shoals and spawn within the shoals, while males of other species, such as *C. beltrani*, establish long-term territories, and the females approach the males in their territories to spawn.

In summary, we provide evidence for pronounced differentiation in social behaviors (shoaling, aggressive behavior, and territoriality) within the phylogenetically young Laguna Chichancanab species flock. Our study in particular highlights the potential role of phenotypic plasticity as a source for behavioral diversification during adaptive radiations within a single lake.

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