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# Learned Social Preference in Zebrafish

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## Summary

**How social aggregations arise and persist is central to our understanding of evolution, behavior, and psychology [1–3]. When social groups arise within a species, evolutionary divergence and speciation can result [4, 5]. To understand this diversifying role of social behavior, we must examine the internal and external influences that lead to nonrandom assortment of phenotypes [6]. Many fishes form aggregations called shoals that reduce predation risk while enhancing foraging and reproductive success [7–9]. Thus, shoaling is adaptive, and signals that maintain shoals are likely to evolve under selection. Given the diversity of pigment patterns among *Danio* fishes [10–13], visual signals might be especially important in mediating social behaviors in this group. Our understanding of pigment pattern development in the zebrafish *D. rerio* [14, 15] allows integrative analyses of how molecular variation leads to morphological variation among individuals and how morphological variation influences social interactions. Here, we use the zebrafish pigment mutant *nacre/mitfa* [16] to test roles for genetic and environmental determinants in the development of shoaling preference. We demonstrate that individuals discriminate between shoals having different pigment pattern phenotypes and that early experience determines shoaling preference. These results suggest a role for social learning in pigment pattern diversification in danios.**

## Results and Discussion

A diversity of communication systems mediate social behavior, and though the functional aspects of these systems have been well studied (reviewed in [17]), little is known of their underlying genetic and molecular mechanisms. The zebrafish system affords us both an organism that engages in a variety of social interactions and a set of developmental and molecular tools particularly well suited to examining the proximate mechanisms responsible for these interactions. For example, a diverse array of adult pigment patterns is exhibited by different *Danio* species, and species with dramatically different pigment patterns cooccur in natural populations [18, 19; California Academy of Sciences Ichthyology Collection catalog numbers CAS134662 and CAS140204]. The pigment patterns of fishes are associated with a variety of behavioral interactions, including shoaling, predation avoidance, species recognition, and mate choice, and such patterns have had important roles in adaptive radiations and speciation [20–24]. In *D. rerio*, numerous single-locus mutants affecting the pigment pattern have been isolated, and several of the corresponding genes now have been identified at the molecular level [16, 25–28]. Such mutants provide an opportunity to dissect the ecological and behavioral significance of pigment patterns at a level not previously achieved. Here, we used a pigment pattern mutant that differs dramatically from wild-type to determine if *D. rerio* exhibits variation in their shoaling preference, if that preference is mediated through visual signals, and what roles internal and external factors play in the acquisition of such a preference.

As a first step in dissecting the behavioral roles of the zebrafish pigment pattern, we compared the shoaling preferences of wild-type fish and *nacre*$^{w2}$ mutants (Figure 1). *nacre* mutants completely lack melanophore stripes owing to a recessive point mutation in *mitfa*, which encodes a basic helix-loop-helix transcription factor that normally acts autonomously to the neural crest-melanophore lineage to specify melanophore fate [16, 29]. We generated families segregating the *nacre* phenotype by backcrossing *nacre* heterozygotes (maintained in the wild-type strain AB$^{UT}$ background) to *nacre* homozygotes. Offspring from these crosses were phenotypically either wild-type (*nacre*/+) or *nacre* mutant (*nacre/nacre*). This design randomizes across effects of other loci that are not linked to the *nacre* mutation.

We sorted subject fish prior to hatching into three treatments: controls (reared with three siblings of the same phenotype), isolates (reared alone), and cross-rears (reared with three siblings of the alternate phenotype). Fish were maintained in these conditions throughout the experiment. When fish developed adult pigment patterns, we tested shoaling preferences by placing individual subject fish ($n = 219$) in a test tank containing separate wild-type and *nacre* stimulus shoals (Figure 2). Prospective shoaling partners were derived from excess siblings of both phenotypes that had been pooled at the outset of the experiment. Each stimulus shoal contained two males and two females that were size matched to the subject. A double pane of ultraviolet-transparent Plexiglas separated each stimulus compartment from the central compartment. The resultant air space blocked any potential chemical or auditory cues. Any variation in preference exhibited by the subject fish would therefore reflect variation in visual signals alone. After allowing fish to acclimate, we recorded the time subjects spent swimming near each shoal during each of two 5 min intervals (methodological details in the Supplemental Data).

We asked first whether zebrafish exhibit a native pref-

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Figure 1. Zebrafish Adult Pigment Pattern

(A) Phenotypically wild-type, heterozygous *nacre* and (B) homozygous *nacre*.

erence for the pigment pattern phenotype of prospective shoaling partners. Our results show that control subject fish exhibited a strong, positive, assortative preference for their own phenotype: wild-type preferred wild-type and *nacre* preferred *nacre* (Figures 3A and 3B). Thus, zebrafish are able to distinguish between alternative pigment pattern phenotypes visually, and there is a strong preference to shoal with individuals of like phenotype. This result suggested either an effect of early environment or a major effect of *nacre* or another closely linked locus on the preference exhibited by subject fish.

We then asked whether the shoaling preference of zebrafish is innate and independent of early environment by rearing subject fish in isolation. Unlike controls, isolates did not show a preference for either phenotype. Wild-type isolates spent 259 ± 84 s (mean ± SD) in association with wild-type shoals and 220 ± 80 s in association with *nacre* shoals (n = 40, $t_{39}$ = 1.83, p = 0.08, paired two-tailed t test). *nacre* isolates spent 243 ± 82 s in association with wild-type shoals and 233 ± 70 s in association with *nacre* shoals (n = 37, $t_{36}$ = 0.53, p = 0.6, paired two-tailed t test). This result suggests that early experience is critical in the development of shoaling preference.

To further elucidate the role of early experience in the acquisition of social preference, we examined fish crossreared with the alternative phenotype: we raised wild-type subjects with *nacre* mutant siblings, and *nacre* mutant subjects with wild-type siblings. Figures 3C and 3D show that crossrearing reverses preferences. Crossreared wild-type subjects exhibited a strong preference for *nacre* mutants, whereas crossreared *nacre* subjects exhibited a strong preference for wild-type. These analyses suggested that rearing treatment, rather than genotype, was the principal determinant of shoaling preference. This role for environment in the acquisition of shoaling preference was further confirmed by factorial analysis of variance: comparisons of time spent with like phenotype revealed significant effects of rearing treatment (control versus cross-reared, $F_{1,138}$ = 29.43, p < 0.0001), but not genotype ($F_{1,138}$, p = 0.7), or genotype by treatment interaction ($F_{1,138}$ = 0.04, p = 0.8; comparing arcsine transformed proportions of time spent with like phenotype over time spent in association with both phenotypes). These results are concordant with an interspecific analysis in which wild-type zebrafish spent less time with other wild-type zebrafish after crossrearing with pearl danios, *D. albolineatus*, though preferences for zebrafish versus *D. albolineatus* were not examined [30]. Our experiments demonstrate that early environment plays a key role in the acquisition of intraspecific shoaling preference in zebrafish.

These shoaling preferences were not due to mate preferences. Our stimulus shoals included both male

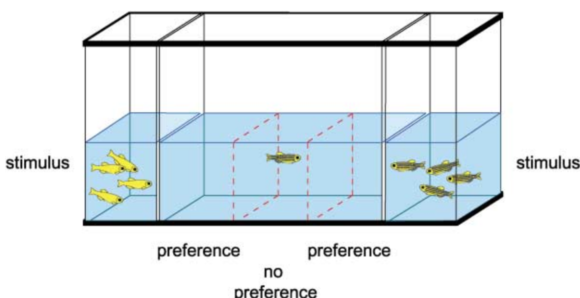


Figure 2. Test Tank

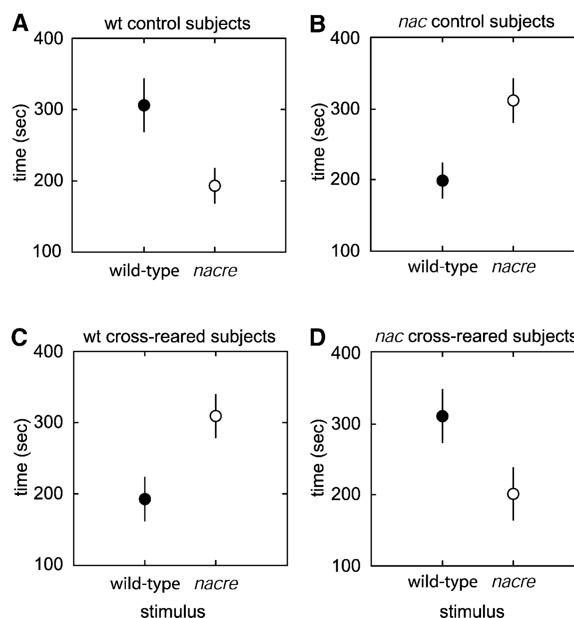


Figure 3. Early Environment Determines Zebrafish Shoaling Preference

(A) Wild-type fish raised with wild-type siblings spent more time in association with wild-type stimulus shoals as compared to *nacre* stimulus shoals (n = 38, $t_{37}$ = 3.04, p = 0.004, two-tailed paired t test).

(B) *nacre* fish raised with *nacre* siblings spent more time in association with *nacre* stimulus shoals as compared to wild-type stimulus shoals (n = 38, $t_{37}$ = 3.41, p = 0.002, two-tailed paired t test).

(C) Wild-type fish raised with *nacre* siblings spent more time with *nacre* stimulus shoals (n = 35, $t_{34}$ = 3.03, p = 0.008, two-tailed paired t test).

(D) *nacre* fish raised with wild-type siblings spent more time with wild-type stimulus shoals (n = 33, $t_{32}$ = 2.19, p = 0.036, two-tailed paired t test). Shown are means ± 95% confidence intervals.

and female fish to simulate naturally occurring mixed sex shoals. Thus, the preference of subjects to associate with one shoal over the other might indicate a mate preference. For example, a female subject might prefer a male in one shoal over the males in the other shoal and therefore spend more time with the shoal containing the preferred male. We therefore repeated these tests by comparing the responses of wild-type females (raised with other wild-type siblings) presented with all-female *nacre* and all-female wild-type shoals, to our wild-type controls (Figure 3A). The response of females to all-female shoals was indistinguishable from that of our wild-type controls to mixed-sex shoals. Wild-type females spent 311 $\pm$ 124 s in association with all-female wild-type shoals and 189 $\pm$ 98 s with all-female *nacre* shoals ($n = 33$, $t_{32} = 3.23$, $p < 0.005$, paired two-tailed t test). Times spent with wild-type shoals did not differ significantly between all-female and mixed-sex stimulus shoals ($n = 33$, $t_{32} = 0.23$, $p = 0.8$, two-tailed t test; comparing arcsine transformed proportions of time spent with wild-type over time spent in association with both phenotypes). Thus, individual behaviors in these assays principally reflect social preference rather than mate preference.

Our results demonstrate that zebrafish exhibit preferences for prospective shoaling partners, that such preferences are mediated by visual signals, and that preferences for specific phenotypes are acquired during development. This is the first time that social preferences and the effect of early environment on social preferences have been shown in a developmental model system. Our knowledge of the genetic and developmental mechanisms underlying the visual signal (i.e., the pigment pattern) offers the opportunity to further dissect how early experience interacts with the development of visual and nervous systems to shape these critical social behaviors in zebrafish and other species. Indeed, the availability of pigment pattern mutants obviates classical methods for altering fish pigment patterns such as freeze branding, dye injection, and video playback. While commonly used, these manipulations are likely to have unpredictable consequences for both the pigment phenotype (given the tetrachromatic nature of zebrafish vision [31]) and the behavioral phenotype (given the small size and fragility of these fish). The results of this study thus set the stage for a complete description of multidimensional preference space [32] and identification of the salient visual elements for shoaling (R.E.E., M.J.R., and D.M.P., unpublished data) by using the stunning variety of pigment patterns provided by zebrafish mutants and double mutants that are one and two mutational steps away from the wild-type pigment pattern [11].

We propose that social preferences of individuals in natural populations also are determined by early experience, as it is highly unlikely that the ability to learn preferences arose de novo in our wild-type $AB^{UT}$ laboratory strain. Learned preferences, therefore, could have substantial evolutionary and behavioral consequences in nature. Zebrafish populations in the wild harbor major-effect mutations including adult pigment pattern variants ([33], and D.M.P., unpublished data). Furthermore, laboratory-derived pigment pattern mutants often resemble the naturally occurring phenotypes of other species [12]. The results of this study demonstrate that pigment patterns serve as visual signals, and early experience with this signal variation determines future social consorts. Thus, a single mutation causes dramatic changes in both the signal and receiver, and thereby constrains the social milieu of an individual to certain genotypes and phenotypes. Since mating is more likely to take place with others in the same social unit, shoaling preferences can promote assortative mating. Thus, even in the absence of specific mate preferences for pigment pattern, shoaling preferences could contribute to genetic divergence. Our analyses of shoaling preference therefore provide a model for how variation at the molecular level can potentially impact population level dynamics and speciation.

**Supplemental Data**

Supplemental Data including Experimental Procedures and exclusion of a UV effect on preference are available at http://www.current-biology.com/cgi/content/full/14/10/881/DC1/.

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# Supplemental Data



# Learned Social Preference in Zebrafish

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**Supplemental Experimental Procedures**

**Variation in Preference**

***Test Tank***

A 122 cm long, 55 cm high, and 32 cm deep all-glass 245 L aquarium was divided into three compartments. The two 25 cm flanking regions were separated from the center by a double pane of UV-transmittant Rhöm Plexiglas GS2458 that was sealed with silicon adhesive to prevent the flow of any water between the panes. The 15 mm airspace between the two Plexiglas panes would block all chemical communication between the compartments and greatly diminish the transmission of auditory cues. The aquarium was lit with a double lamp, 125 cm long, fluorescent fixture (lamped with one 40 W cool blue tube and one 40 W Reptical tube). The tank was covered on sides and back with translucent plastic sheeting. Washed gravel was used as a substrate covering the bottom of all three compartments. The aquarium was filled with water to the 25 cm level. The water temperature was maintained at 29°C with a submersible Ebo-Jager 100 W heater that was removed during testing. The two 25 cm flanking areas of the inner compartment were marked on the exterior of the glass with a black grease pencil to demarcate the left and right preference areas (see Figure 2 in the main text).

***Fishes***

We used fish either heterozygous for the *nacre*$^{w2}$ (*nacre*) allele, exhibiting the wild-type pigment pattern phenotype (see Figure 1 in the main text), or homozygous for the *nacre* allele, exhibiting the mutant phenotype. The *nacre* mutant lacks melanophores and exhibits a pigment pattern dramatically different from the wild-type. This recessive phenotype results from an A to T transition that yields a premature stop codon in the locus coding for the *mitfa* transcription factor, which normally acts autonomously to the melanophore lineage to specify melanophore fate [S1]. To produce five sibships for use in this experiment, we generated crosses segregating the *nacre* mutant phenotype in the inbred mapping strain AB$^{UT}$ background (thereby randomizing across potential effects of other unlinked loci). Wild-type (*nacre*/+) and *nacre* mutant (*nacre*/*nacre*) siblings were sorted into one of three different treatments: control raised with three sibs of the same phenotype, isolates reared alone, and cross-rears raised with three siblings of the alternate phenotype. The embryonic *nacre* phenotype [S1] allowed us to sort the embryos into these treatments at 60 hr post fertilization.

*Control*. Larvae were placed in transparent 100 ml plastic cups with three fish of the same phenotype. This cup was suspended in a 1 L plastic aquarium, whose sides are covered with a translucent plastic sheet on three sides to prevent the fish from observing fish in adjacent tanks. Two holes in opposite sides of the cup with screen glued over them allowed water to flow through the cup.

*Isolate*. Larvae were placed in an apparatus identical to that described above, but without any other fish.

*Crossrear*. Larvae were placed in an apparatus identical to that described above, but three fish of the alternate phenotype (wild-type for homozygotes, mutant for heterozygotes) were placed in the cup.

In all treatments, when the fish reached 1 cm standard length, they were transferred from the cup to the aquarium. Excess fish from the crosses were pooled and raised for use as stimulus fish in the preference assay. We combined both heterozygous and homozygous individuals in these stock tanks, resulting in stimulus fish that had experienced both phenotypes.

***Preference Assay***

We measured shoaling preference of the subject fish as follows. At 90 days postfertilization, all subject fish had attained at least 1.5 cm standard length. Fish were then chosen at random from the available subjects and used in the preference assay. Opaque plastic barriers were placed at either end of the central portion of the test tank. A shoal of four wild-type (two male and two female, matched in size to the subject) were chosen at random from the pooled stocks and placed in one stimulus compartment (side determined by coin toss). A shoal of four *nacre* fish (two male and two female, matched in size to the subject) were placed in the other stimulus compartment. The subject fish was placed in the central compartment. Shoals of as few as four zebrafish exhibit shoaling behavior indistinguishable from larger groups [S2].

The fish were allowed 10 min to acclimate to the tank. The barriers were then removed and the subject fish was given the next 15 min to recognize both stimulus shoals. Recognition was defined as parallel swimming with a member of the stimulus shoal. The time needed for the fish to recognize both stimuli was noted as the latency. If the subject did not recognize both shoals in 15 min, the test was aborted. During the following 5 min, the time spent by the subject in either preference area was noted. The barriers were then replaced, the stimulus shoals were exchanged to control for side bias, and the above steps were repeated. The association times noted in these two 5 min intervals were combined in the analysis shown in Figure 3 of in the main text. All fish were tested by 160 days postfertilization, and days postfertilization had no effect on the analysis.

**All-Female Preference Test**

The methods were identical to the previous test except as follows.

***Fishes***

Wild-type subject fish were sorted into cups with three of their wild-type siblings, and only female subjects were used in the assay.

***Preference assay***

The stimulus shoals contained only female fish. All tests were completed by 135 days postfertilization.

**UV Effect**

As full-spectrum lighting was not used in the test tank, we tested for the possible existence of a UV effect on shoaling preference. The test tank and protocol were identical to that used by Cummings et al. [S3], with the following exceptions. The subject fish were wild-type zebrafish raised with wild-type siblings, and the stimuli were shoals of four wild-type zebrafish (two male and two female, matched in size to the subject). No UV effect was found (mean time $\pm$ SD spent with UV+ shoals 253 $\pm$ 105 s, with UV shoals 219 $\pm$ 94 s, n = 50, $t_{49} = 1.315$, p = 0.20, two-tailed paired t test).

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