

between conspecifics and other co-occurring minnows, such as members of the genera *Barilius*, *Danio*, *Devatio* and *Puntius* (Engeszer et al., 2007).

Previous work on shoaling in the zebrafish has focused on the propensity of individuals to shoal (Wright et al. 2003) and the choice of shoalmates (Mann et al. 2003). We showed that zebrafish express a visually mediated shoaling preference and that early environment determines the preferred phenotype (Engeszer et al. 2004): wild-type zebrafish preferred either other wild-type zebrafish, or a stripeless mutant, *nacre*, depending on which phenotype they experienced during development (Fig. 1a, b).

In this study, we asked three critical questions for understanding shoaling in wild-type zebrafish. When do zebrafish begin shoaling? When is the visual preference first shown? And does this preference change over the fish's life as it experiences other phenotypes, or, is it immutable once established? We find that shoaling begins relatively soon after hatching. By contrast, a visual preference for shoalmates is not shown until the later juvenile stages. Furthermore, this preference does not change even in response to prolonged exposure to alternative visual phenotypes.

METHODS

Overview

We raised small groups of wild-type fish (Fig. 1a) in visual isolation from other fish in the laboratory. When these wild-type fish reached a particular developmental stage, we assayed their propensity to shoal and their shoaling preference using a binary preference test. One subject was chosen at random from the group, placed in a test tank (Fig. 2) and presented with a shoal of wild-type fish

and a shoal of *nacre* fish that lack melanophore stripes (Lister et al. 1999; Fig. 1b). The subject was separated from each stimulus shoal by a transparent barrier. We recorded each subject's time in association with each shoal. We tested fish at several development stages ranging from freshly hatched larvae to adults.

To assess the stability of shoaling preference, we assayed the visual preference of juvenile wild-type zebrafish. Each subject had been raised with three wild-type siblings. We tested them in the same tank and using the same protocol described above. We then manipulated the social environment of these subjects for an additional 30 days, housing half with wild-type shoalmates and half with *nacre* shoalmates. We then retested the subjects to see if their preference had changed.

Fish Stocks and Rearing Conditions

Stocks

We backcrossed *nacre*^{w2} heterozygous fish in the AB^{WP} background to *nacre* homozygotes to obtain sibships that contained half wild-type individuals and half *nacre* mutant individuals (Fig. 1a, b; for zebrafish stock naming conventions, see <http://zfin.org>); *nacre*^{w2} is a recessive, single-locus mutant phenotype arising from a mutation in *microphthalmia-a*, which encodes a transcription factor essential for melanophore development (Lister et al. 1999). Shoaling behaviours shown by *nacre* mutant fish are qualitatively and quantitatively indistinguishable from wild type (Engeszer et al. 2004; personal observations).

Onset of shoaling and preference

Fish from these *nacre* backcrosses were then sorted into either subject treatments, from which we later chose

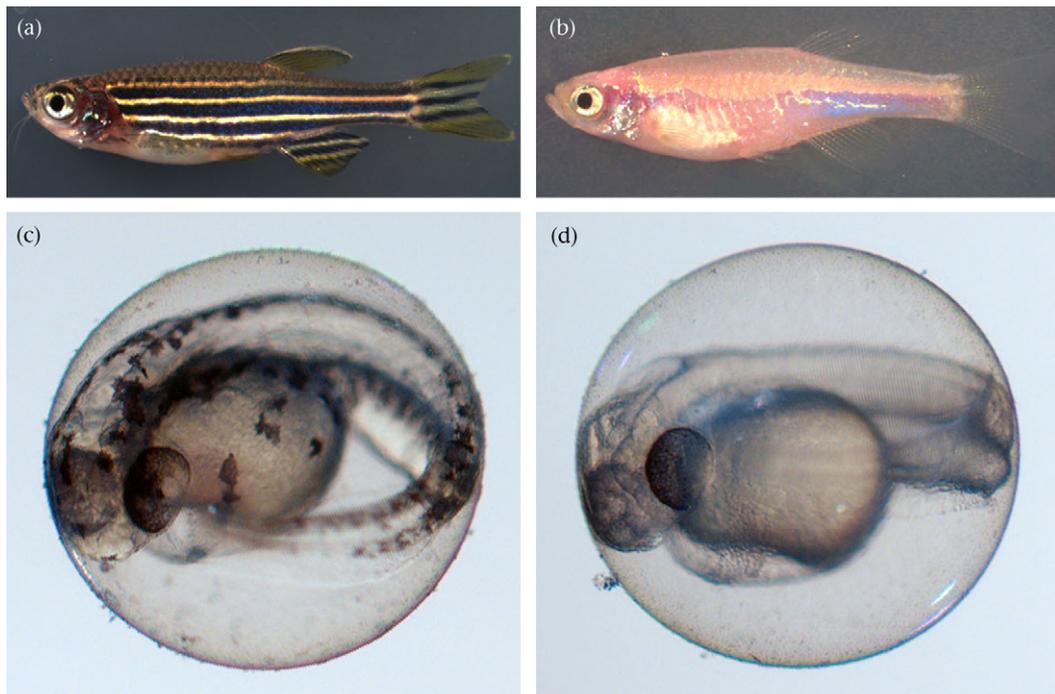


Figure 1. Adults: (a) wild-type pigment pattern; (b) *nacre* mutant pigment pattern. Embryos: (c) wild-type phenotype; (d) *nacre* mutant phenotype.

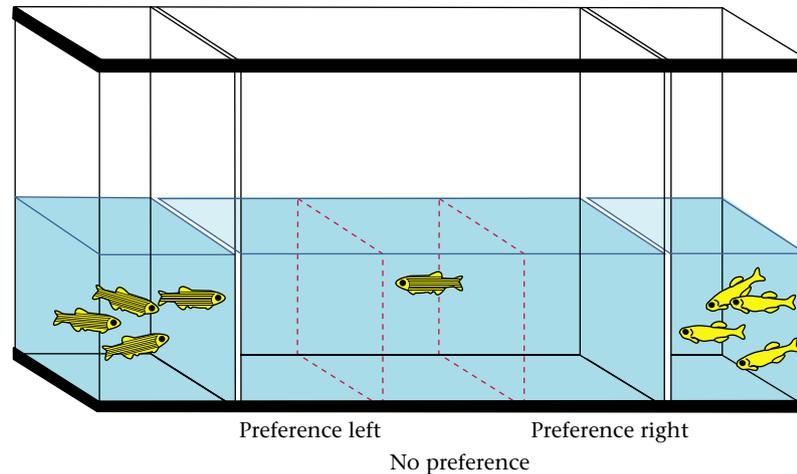


Figure 2. Schematic of the test tank. The dashed lines mark the interior boundaries of the preference areas. The double lines show both the position of double panes of Plexiglas and the outer boundaries of the preference areas. Stimulus shoals are shown in each of the outermost compartments and the subject is in the central 'no preference' area.

individuals as subjects for analysing shoaling behaviour, or stimulus treatments, from which we chose fish to act as members of stimulus shoals. As *nacre* mutants have an embryonic pigment pattern phenotype different from the wild type (Fig. 1c, d), we were able to sort individuals into treatments before their hatching. Wild-type fish were raised in groups of four, with one fish from each group chosen randomly as a subject for analysis. Stimulus fish were raised in groups of four, of which two were wild type and two were *nacre* mutants; we chose fish from these treatments for use in the stimulus shoals. Rearing the stimulus fish under the same conditions as the subject fish controlled for environmental effects that might cause them to grow at a rate different from their subject siblings, whereas rearing them as mixed wild type and *nacre* groups eliminates any shoaling bias they might otherwise express (Engeszer et al. 2004). We raised these groups in opaque cups in which they were visually isolated from any fish not in their treatment until they reached one of the following stages (Snyder & Muth 2004) in chronological sequence.

Preflexion stage. Individuals are approximately 3.5 mm standard length (SL) and are characterized by a continuous fin fold, straight notochord and no fin rays.

Early flexion stage. Individuals are approximately 6 mm SL and are characterized by the upturn of the posterior notochord (urostyle) and the appearance of the first caudal fin rays.

Postflexion stage. Individuals are approximately 7 mm SL and have completed flexion, well-developed hypurals, and a bilobate swimbladder (rather than the single-lobed swimbladder seen at earlier stages).

Metalarvae stage. Individuals are approximately 8.5 mm SL and have nearly complete median fins and pelvic fin buds.

Juvenile stage. Individuals are approximately 10 mm in SL, are not yet reproductively active and have complete fins, complete squamation and a nearly complete adult pigment pattern.

Adults stage. Individuals are over 15 mm SL and have developed gonads.

We tested 20 subject fish at each stage; individual fish were tested only once.

Plasticity of preference

Forty wild-type juveniles that had each been raised with three wild-type siblings (as described for subjects above) were tested for preference (see **Test Protocol** below). We then placed individuals at random into one of two social environments, control or cross-rear. We placed each of the 20 control fish in a 2.8-litre Aquaneering flow-through tank with three other wild-type zebrafish as social models. Model and subject fish were siblings matched in size and age. We covered the sides of each tank with translucent white plastic to obscure the view of fish in any nearby tanks. We placed each of the cross-rear subjects in identical tanks, containing three *nacre* siblings as social models. We kept subjects in their treatment tanks for 30 days and then retested them. Although subjects and model fish were siblings, they had no prior experience with one another. The fish used as shoaling stimuli in the experiments were also siblings of the subjects, but the test subjects had not previously seen any of the stimulus fish.

Test Tanks

Adult fish examined for both onset and plasticity of shoaling preference were tested in a 245-litre all-glass aquarium (122 × 55 × 32 cm, length × height × depth) that was divided into three compartments. The two flanking regions (25 cm) were separated from the centre 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 airspace (15 mm) between the two Plexiglas panes blocked all chemical communication between the compartments and greatly diminished 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 neutral grey photographic paper. 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 (Fig. 2).

The preflexion, early flexion, postflexion, and metalarval stages were tested in a tank (22.5 × 9.5 × 5.5 cm) made of 2-mm-thick UV transparent Plexiglas and filled to a depth of 4.5 cm. The exterior sides were covered with neutral grey photographic paper. The tank was divided into stimulus and preference areas that were the same proportions as the adult tank. Thinner Plexiglas was used in place of the Rhöm Plexiglas to separate the preference areas from the stimulus areas. The tank was otherwise similar to the tank for adults.

The juvenile stage subjects were tested in a 40-litre all-glass aquarium (40.5 × 21 × 24 cm) filled to a depth of 18 cm. The sides were covered with neutral grey photographic paper and a finer sand substrate was used. The tank was divided into stimulus and preference areas that were the same proportions as the adult tank. The tank was otherwise similar to the tank for adults.

Test Protocol

Fish were kept in their treatments until they reached one of the stages described above, at which time we measured shoaling preference as follows. 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 fish was placed in one stimulus compartment (side determined by coin toss). A shoal of four *nacre* fish was placed in the other stimulus compartment. All stimulus fish were chosen from stimulus treatments and matched in size and stage to the subject. We used four zebrafish in the stimulus shoals because groups of this size show shoaling behaviour indistinguishable from that of larger groups (Breder & Halpern 1946). The subject fish was placed in the central compartment. When the subjects were adults, the stimulus shoals included two female and two male fish; at earlier stages we were unable to sex the stimulus fish.

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. We carried out tests from late morning through the afternoon, and time of day had no effect on the analysis (data not shown).

Analysis

Onset of shoaling and preference

We compared the time spent in the 'preference' areas across stages to ascertain the onset of shoaling. We expected fish that did not shoal to spend less time in the 'preference' areas than fish that did shoal with the stimulus fish. We performed an arcsine transform on the proportion of time the subjects spent in the 'preference' areas to the total test time (600 s) to assess the onset of shoaling behaviour. The results were then compared between stages using a one-way ANOVA, and the means were compared using Tukey–Kramer honest significant difference test (HSD).

We then compared the time spent in association with either stimulus to determine whether subjects showed a preference. We compared the time spent with the reared stimulus with the time spent with the nonreared stimulus using Student's *t* test for each stage.

Plasticity of preference

To assay any change in shoaling preference between the first time the fish were tested and the second, we compared the proportion of time spent with the wild-type stimulus in each test. We used the arcsine transform of this proportion in a paired Student's *t* test to determine any significant difference between the amounts of time spent associating with wild-type shoals.

RESULTS

Onset of Shoaling and Preference

Because fish vary tremendously in the ontogenetic timing of shoaling behaviour (see references above), we analysed whether zebrafish shoal immediately on hatching or whether this behaviour arises during later development. The two hypotheses that we considered were (1) fish shoal immediately on hatching or (2) fish begin to shoal at some point during posthatching development.

Our analyses revealed a clear ontogeny for shoaling behaviour, from relatively sessile, early stage preflexion larvae to later stage, postflexion larvae, whose propensity to shoal is indistinguishable from that of adults. Preflexion larvae did not respond in the protocol, remaining immobile during the test, and so are not included in the analysis. The time that individuals spent shoaling differed significantly between stages (ANOVA: $F_{1,4} = 5.23$, $P < 0.001$; Fig. 3). Nevertheless, shoaling times for postflexion, metalarvae, juveniles and adults did not differ significantly from one another and all of these except metalarvae differed significantly from early flexion larvae (Tukey–Kramer HSD: $q^* = 2.79$, $\alpha = 0.05$). Thus, shoaling behaviour arises during

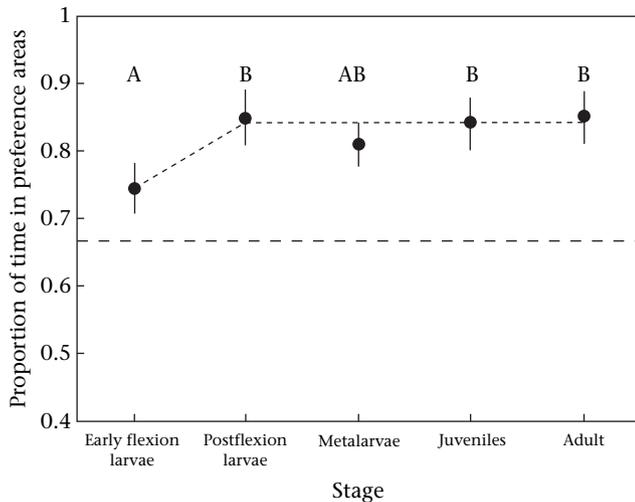


Figure 3. Onset of shoaling. The proportion of time spent in the preference areas relative to the total observation time ((time left + time right)/600 s) is shown for each stage. The dashed line represents the null expectation of 66% of the time spent in the preference areas. Circles represent the means for each stage (back transformed from the data used for the ANOVA), and error bars represent the 95% confidence intervals. Early flexion larvae, $N = 19$; postflexion, $N = 20$; metalarvae, $N = 16$; juvenile, $N = 20$; adults, $N = 20$. The letters A and B designate groups that were significantly different (Tukey–Kramer HSD test: $\alpha = 0.05$).

larval development, and zebrafish continue to shoal from this point onwards.

Having identified the onset of shoaling behaviour, we asked when visual preferences are first expressed. We considered the following hypotheses: (1) fish show a visual shoaling preference as soon as they begin to shoal or (2) fish begin to express a visual shoaling preference at some later developmental stage. To distinguish between these hypotheses, we raised fish with their wild-type siblings to various stages (see [Methods](#) for stage descriptions) and gave them a choice between associating with either wild-type or *nacre* shoals.

Our data show that although larval zebrafish show shoaling behaviour, they do not discriminate between the stimulus shoals on the basis of visual signals. Juveniles, however, show a robust, visually mediated preference. The juvenile and adult stages showed a significant difference in the amount of time spent with the reared stimulus (two-tailed paired Student's t tests: juvenile: $N = 20$, $t_{19} = -2.83$, $P = 0.01$; adults, $N = 20$, $t_{19} = -2.4$, $P < 0.05$), whereas none of the other stages did (early flexion larvae: $N = 19$, $t_{18} = 0.253$, $P = 0.8$; postflexion, $N = 20$, $t_{19} = -0.98$, $P = 0.3$; metalarvae, $N = 16$, $t_{15} = 0.065$, $P = 0.95$; [Fig. 4](#)). Therefore, the onset of shoaling and the first expression of the visually mediated preference are decoupled, and the preference is shown long after shoaling begins.

Plasticity of Preference

We know that early life experience plays a key role in the acquisition of shoaling preference ([Engeszer et al. 2004](#)), but we did not know if changes in the subsequent

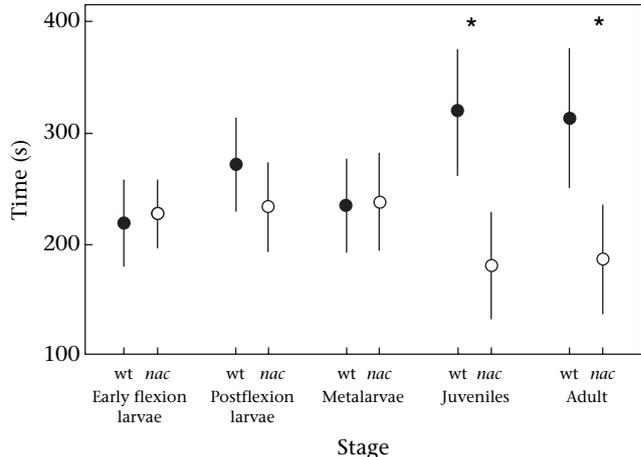


Figure 4. Onset of preference. For each stage, the mean time in association with both wild-type (wt, ●) and *nacre* (*nac*, ○) shoals is shown. Error bars represent the 95% confidence intervals. Early flexion larvae, $N = 18$; postflexion, $N = 20$; metalarvae, $N = 16$; juveniles, $N = 20$; adults, $N = 20$. Asterisks denote stages showing a significant preference.

social environment might cause changes in this shoaling preference. Does the preference remain labile throughout the life of the fish, or is the preference immutable once it has been established? After rearing zebrafish with wild-type shoalmates and testing their preference, we then manipulated their social environment, housing half with wild-type shoals (controls) and half with *nacre* shoals (cross-rears) for one month.

Even with prolonged exposure to other fish showing a dramatically different pigment pattern, the preference of subject fish for wild-type shoals did not change. Neither control nor cross-reared subjects showed a significant change in the proportion of time they spent with the wild-type stimulus shoal (two-tailed paired Student's t tests: controls: $N = 18$, $t_{17} = 0.051$, $P = 0.57$; cross-rears: $N = 15$, $t_{14} = 1.650$, $P = 0.17$). Thus, the visually mediated shoaling preference was stable in this assay and may be resistant to change during later life.

DISCUSSION

This work shows that zebrafish shoal relatively early in postembryonic development, that they show a visual preference for certain shoalmates much later in development, and that this preference appears immutable once it has been established. At very early stages, zebrafish do not shoal, as seen in the failure of preflexion larvae to respond to the protocol. This lack of response is hardly surprising. Preflexion larvae spend their time adhering to nearby surfaces via their adhesive organs and swim only when startled. Early flexion larvae, while far more mobile than preflexion larvae, spent significantly more time in the “no preference” area than all of the later stage larvae with the exception of the metalarvae. These data do not exclude the possibility that early flexion larvae are shoaling, but they do show that shoaling occurs very early in postembryonic

development and, by the postflexion stage, zebrafish are clearly shoaling.

The relatively late appearance of the visually mediated preference is interesting given the relatively early onset of shoaling behaviour. Although juveniles and adults show a robust preference for the reared stimulus phenotype, earlier stages do not. Zebrafish possess a functional visual system at the preflexion stage (Schmitt & Dowling 1999). Preflexion larvae can recognize food (Kimmel et al. 1995) and respond to shadows passing over them (Easter & Nicola 1996), but the full complement of photoreceptors is not expressed until approximately 12 days postfertilization (Branchek & Bremiller 1984). This timing roughly coincides with the transition from preflexion larva to postflexion larva (R. E. Engeszer, unpublished data). At this point in development, all of the receptor types are present and functioning in the retinal mosaic, which continues to grow in size as the zebrafish grow to adulthood (Branchek & Bremiller 1984). Ontogenetic changes in the visual system during the larval stages and through metamorphosis could, therefore, explain the late onset of the visually mediated preference. Alternatively, changes in the higher processing of visual signals in the optic pathways of the brain may account for the ontogenetic variation in the preference.

The appearance of the preference also coincides with the emergence of the postmetamorphic, or adult, pigment pattern (Parichy & Turner 2003). The larval pigment pattern of three melanophore stripes, one dorsal, one medial and one ventral, is conserved throughout the close relatives of zebrafish (Quigley et al. 2004; Parichy 2006). This larval pigment pattern is further conserved throughout the cyprinids and is even found in the related Catostomidae (Snyder & Muth 2004). Zebrafish inhabit waters with a number of other cyprinids and one, *Puntius shaly-nius*, spawns in rice paddies in which zebrafish larvae and juveniles are found (Engeszer et al. 2007). In situations where larval cyprinids co-occur, visual cues would be a poor indicator of conspecific shoalmates. Unlike their larvae, adult danios and, more broadly, adult cyprinids, show dramatically different pigment patterns (Quigley et al. 2005). The late onset of the visually mediated shoaling preference, thus, co-occurs with the stage at which zebrafish would be able to visually distinguish between conspecific and heterospecific fish.

Further observations regarding zebrafish life history lend insight into possible selective advantages of a late onset shoaling preference. Larval and juvenile zebrafish inhabit rice paddies and seasonal waters that may provide refuge from predators (Engeszer et al. 2007) and the selective pressures on these fish to shoal only with fish of a similar phenotype may be greatly reduced. The onset of the preference coincides with the stage at which fish are leaving these refuges and coming into contact with piscine predators. At this point, the oddity effect (Landeau & Terborgh 1986) implies a significant survival cost to individuals for shoaling with fish of a different phenotype, as individuals that do so will be preferentially preyed on. Changing selective regimes as these fish move from one area to another may account for the late appearance of the visual preference.

Given the importance of the early social environment in the determination of the visual preference, larval zebrafish must either be using a different aspect of the visual signal, such as overall size of the shoaling fish, or an alternative sensory modality to assess shoalmates. Strong evidence exists for the use of olfactory cues in fish in general and zebrafish in particular in the identification of conspecifics (McLennan & Ryan 1997) and even relatives (Mann et al. 2003). If larval zebrafish identify conspecifics using a different sensory modality, they would associate with conspecifics and be exposed to their pigment pattern during preference acquisition.

Finally, our results suggest that the visual preference of zebrafish may be immutable once it is established. Individuals raised with wild-type fish and later cross-reared with *nacre* fish did not lose their preference for wild-type shoals. Because the adult pigment pattern develops rather late, the subject fish had less than a month of exposure to it before they were tested and shifted into their new social environment. Yet even spending a month with extremely different social consorts did not significantly change the preference shown. This result, coupled with early cross-rearing experiments, strongly suggests that there exists a critical period during which the visually mediated shoaling preference is acquired, after which time it is unchangeable. This situation is reminiscent of the process of song learning in birds (Marler & Tamura 1964). Social behaviour in zebrafish could provide a fascinating parallel system for the study of this type of learning and the arsenal of tools available to investigate changes at the neurological and molecular level in zebrafish could make the investigation of the mechanisms underlying the establishment of this visual preference particularly exciting.

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