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Assortative preferences for stripes in danios

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Decisions about joining social groups are often contingent on one's own phenotypic characteristics and those of existing group members. Zebrafish and their allies (genus *Danio*) vary substantially in the expression of conspicuous lateral stripes. We presented wild-type zebrafish (*D. rerio*), two artificially selected *D. rerio* strains with reduced stripe expression, as well as the stripeless *D. albolineatus* and the striped *D. nigrofasciatus*, with simultaneous choices between synthetic, computer-animated shoals of stripeless and striped *Danio*. Each species and strain of *Danio* preferred the phenotype that most resembled its own, with a strong preference for striped stimuli by fish with dark-stripe phenotypes, and a strong preference for no stripes by fish lacking stripes. Stripes are therefore a key shoaling cue in *Danio*. Individuals appear to acquire a general preference for stripes, or no stripes, as a consequence of experience with a specific stripe phenotype.

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The formation of social aggregations is the product of behavioural decisions made by multiple individuals. The decision of with whom to associate is among the most fundamental an individual can make, influencing its reproductive success (Isvaran & St Mary 2003), foraging rate (Metcalfe & Thompson 1995) and risk of death (Landeau & Terborgh 1986). A notable form of social aggregation is shoaling in teleost fish. Shoals minimize predation risk through increased vigilance and dilution of prey, and membership in a shoal can facilitate foraging and provide opportunities for mating (Pitcher & Parrish 1993).

Shoaling decisions often depend on the phenotypic characteristics of existing group members. Individuals often assort with respect to characteristics such as body size (Hoare et al. 2004; Wong & Rosenthal 2005), colour (McRobert & Bradner 1998), species (Barber et al. 1998; Krause et al. 2000) or individual familiarity (Barber & Wright 2001). Assortative shoaling on the basis of such attributes is thought to reduce predation risk by minimizing phenotypic oddity (Peuhkuri 1997) or coordinating antipredator behaviour (Chivers et al. 1995). Assortative shoaling can also minimize competition with group members (Landeau & Terborgh 1986; Peuhkuri 1997; Ward et al. 2002).

Zebrafish (Cyprinidae: *Danio rerio*) are so named because of the conspicuous, dark lateral stripes that characterize

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adults. In nature, *D. rerio* shoal in small groups of 2–10 fish (Pritchard et al. 2001), and stripes may be critical for identifying shoalmates. Furthermore, there is broad overlap in the ranges of different *Danio* species, and stripe patterns are fairly stereotyped within species in the wild (Talwar & Jhingran 1991; Engeszer et al. 2004). Stripes may thus be used to discriminate conspecifics from heterospecifics. The expression of stripes is, however, highly labile among species: there is substantial variation among closely related taxa in the form, number, and presence of stripes (Talwar & Jhingran 1991; Engeszer et al. 2004). There are also numerous, viable mutant lines and artificially selected strains of stripeless *D. rerio* (Quigley & Parichy 2002). Such stripeless mutants were recently used to show that early experience determines shoaling preference (Engeszer et al. 2004). Wild-type individuals exposed to stripeless *nacre* mutants early in ontogeny preferred to shoal with *nacre* individuals as adults, and vice versa. While *nacre* is a single-locus mutation, it is difficult to exclude the possibility that subjects were attending to pleiotropic effects on motor patterns or other cues in making their choices.

Do the conspicuous stripes of zebrafish serve as a shoaling cue? We used playback of computer animations to isolate the effect of the conspicuous lateral stripes in *Danio* varying in stripe phenotype. Zebrafish have been shown to respond to playback of computer animations (Turnell et al. 2003). We tested visual preference for striped versus stripeless shoalmates in two striped species, wild-type *D. rerio* and *D. nigrofasciatus*, as well as in three lineages lacking conspicuous dark stripes: *D. albolineatus*

and two mutant strains of *D. rerio*, *golden* and *leopard* (Fig. 1). *Danio albolineatus* and *D. nigrofasciatus* are close relatives of *D. rerio* (Parichy & Johnson 2001).

MATERIALS AND METHODS

Stimulus Construction

We constructed a synthetic animation of shoaling danios as described in Rosenthal (2000). Stimuli were modified versions of those used in Turnell et al. (2003). All modelling, animation and rendering were done using 3D Studio Max 1.0 (Kinetix, Burlingame, California, U.S.A.) on a Dell Optiplex GXPro computer, using a Targa 1000 board for digital-to-analogue conversion of video signals and vice versa. We used the program to construct a three-dimensional model stimulus based on a photograph of an adult *D. rerio*, which provided the basis for the lateral outline, fin shape, and colour and pattern properties. We measured body thickness at three points (orbit, anterior dorsal fin insertion and caudal peduncle) in 10 adult *D. rerio* from our laboratory colony to generate cross-sections for the body.

To animate the stimulus, we placed three *D. rerio* in an aquarium and videotaped normal shoaling behaviour. The aquarium was scaled to the dimensions of the playback monitors so that the stimulus would ultimately appear life-sized. The three-dimensional model was superimposed over a single exemplar swimming across the aquarium, reversing direction and swimming back to its original location. Model parameters were manually adjusted to match the position, orientation and deformation of the exemplar fish. The resulting 8-s sequence was looped to create a 5-min stimulus sequence. The apparent size of the fish on the playback monitor was scaled using the mean standard length (snout to caudal peduncle) of 10 adult *D. rerio* from our laboratory colony. Danios shoal in groups of several individuals, so we twice duplicated the stimulus fish. For the first duplicated fish, we scaled body size up by one standard deviation, offset the time track by 1 s, and moved the fish so that it would appear to be slightly in front of the original stimulus. For the second, body size was reduced in scale by one standard deviation, the time track was offset by 2 s from the original, and the fish was moved slightly back from the original exemplar. This technique produced the appearance of three distinct, shoaling fish, providing subjects with stimulus shoals within the natural shoal size range of 2–10 individuals (Pritchard et al. 2001).

We used a homogeneous neutral grey background (red/green/blue values all set to 127). Apparent illumination was designed to simulate natural light conditions at midday in a shallow freshwater environment, with directional white light coming from above the stimuli, and reflected and scattered in all directions below and to the sides of the stimulus fish.

The base 'striped' stimulus (Fig. 1, upper left) was subsequently manipulated to produce a 'stripeless' stimulus (Fig. 1, lower left), identical to the base stimulus except for colour and pattern. The digitized photograph of *D. rerio* used to provide colour and pattern cues was modified using Adobe Photoshop: blue stripes were erased and replaced with background body colour.
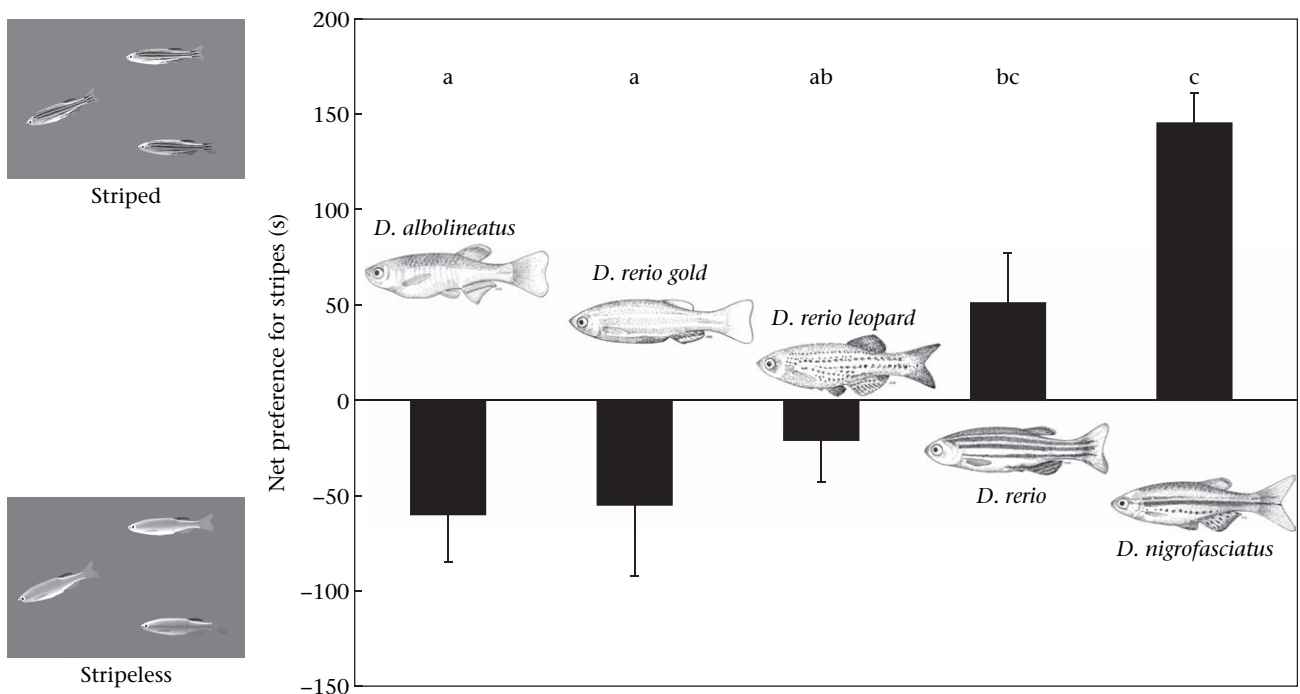

Figure 1. Net preference of each phenotype for stripes ($\overline{X} \pm$ SE association time in seconds). A negative value indicates a preference for the stripeless stimulus. Letters above bars indicate significantly different groups in ANOVA. Representative stills from stimuli used in video playback are shown at left.

Subjects, Playback and Data Analysis

Wild-type (striped) *D. rerio*, *golden* strain *D. rerio*, *leopard* strain *D. rerio*, and *D. albolineatus* were obtained from Amazonia Aquariums (Austin, Texas, U.S.A.), and *D. nigrofasciatus* were provided by D. Parichy. All fish were sexually mature adults and had been reared exclusively with members of their own species or strain. Testing procedures largely followed Engeszer et al. (2004). Subjects were tested in a 76.8×31.8-cm aquarium filled to a height of 25.1 cm. The aquarium was divided into three sections: left and right 'choice' sections 10 cm wide and abutting the ends of the aquarium, and a 56.8-cm neutral section in the centre. A Panasonic TR-930B 9-inch monochrome monitor abutted either end of the aquarium. Monitor output was matched before each test by placing the monitors adjacent to each other, playing identical sequences, and then adjusting the controls until the images were indistinguishable. Presentation sequences were played on Panasonic AG-1970 S-VHS video-cassette players. Subjects were acclimatized in the test tank with the monitors on for 10 min before the start of each test. We presented animals with 10 min of monochromatic screen on both sides followed by simultaneous presentation of the 300-s test stimuli: 'striped' on one side and 'stripeless' on the opposite side. The subjects were then presented with a 10-min monochromatic screen interval followed by the same set of stimuli presented on opposite sides of the set-up. We recorded the time that the fish spent in each section for the duration of each stimulus period. Side biases were controlled across subjects by alternating the location of the first stimulus from one trial to the next. Each subject was tested only once.

For each species or strain, we performed a paired *t* test to evaluate preference for striped versus stripeless. Our a priori prediction, based on Engeszer et al.'s (2004) results, was that the striped forms (wild-type *D. rerio* and *D. nigrofasciatus*) would prefer the striped animation, and that stripeless forms (*golden* strain of *D. rerio* and *D. albolineatus*) would prefer the stripeless animation. Statistical tests for these comparisons were accordingly one tailed. No a priori prediction was made for the intermediate *leopard* strain of *D. rerio*. We then performed a one-factor ANOVA, with species or strain as the categorical variable, on the arcsine square-root transformed proportion of time spent with the striped stimulus relative to the stripeless stimulus. We used Fisher's least significant differences test to evaluate pairwise differences in response.

RESULTS

Subjects shoaled preferentially with a stripe phenotype resembling their own (Fig. 1). The striped wild-type *D. rerio* and *D. nigrofasciatus* showed strong preferences for stripes (*D. rerio*, $t_{18} = 1.96$, $P = 0.033$; *D. nigrofasciatus*, $t_{15} = 2.37$, $P = 0.016$). The stripeless *D. albolineatus*, in contrast, significantly preferred to shoal with stripeless animations ($t_{24} = 2.38$, $P = 0.013$) and a similar but nonsignificant tendency was found in *golden* mutants of *D. rerio* ($t_{20} = 1.46$, $P = 0.079$). *Leopard* mutants of

D. rerio, which have an intermediate, mottled pattern, showed no significant preference ($t_{17} = 0.93$, $P = 0.365$).

There was a significant effect of species or strain on stripe preference (one-way ANOVA: $F_{4,103} = 4.18$, $P = 0.004$; Fig. 1). 'Gold' *D. rerio* showed a significant difference in response from wild-type conspecifics (Fisher's LSD test: $P = 0.016$; Fig. 1) but were not significantly different from the stripeless *D. albolineatus* ($P = 0.809$). Both striped wild-type species, *D. rerio* and *D. nigrofasciatus*, showed significantly stronger preferences for stripes than did the unstriped *D. albolineatus* or gold *D. rerio* (Fig. 1).

DISCUSSION

Each species and strain of *Danio* preferred the phenotype that most resembled its own, with a strong preference for striped stimuli by individuals with dark-stripe phenotypes, and a strong preference for no stripes by individuals lacking them. 'Leopard' danios, with an intermediate stripe pattern, were intermediate in preference. Like other shoaling fish (Barber et al. 1998; McRobert & Bradner 1998; Krause et al. 2000; Hoare et al. 2004), *Danio* show a distinct preference for associating with individuals that are similar in appearance to themselves. Furthermore, the preference appears to depend in large part on a single, iconic cue: the stripe pattern.

Assortative shoaling in *Danio* is likely to result in assortative mating (Engeszer et al. 2004). There was no discernible difference in how preferences varied among species with respect to how they varied among *D. rerio* phenotypes. Our results thus suggest that assortative preference with respect to stripes may influence both the origin and maintenance of reproductive isolation. Selection may act strongly against 'odd' stripe phenotypes. Even though viable, stripeless mutants of *D. rerio* are readily obtained in the laboratory, these may be at a severe disadvantage in the wild because of both heightened vulnerability to predators and reduced association with potential mates.

Engeszer et al. (2004) recently showed that both wild-type and *nacre* mutant *D. rerio* shoal assortatively by phenotype, and that this shoaling preference depends on previous visual experience; wild-type fish raised with *nacre* siblings spent more time shoaling with *nacre* mutants, and vice versa. Among species, *D. rerio* cross-reared with *D. albolineatus* spent less time with conspecifics than did controls (McCann & Carlson 1982). It is likely, therefore, that the assortative preferences seen in our experiment depend on previous experience with individuals expressing similar stripe patterns. This experience could be the result of imprinting during an early critical period (Nevitt et al. 1994) or of recent familiarity with similar individuals (Barber & Wright 2001).

A compelling implication of our results is that in *Danio*, imprinting results in visual generalization (Stach et al. 2004): a general preference for stripes, or for no stripes, emerges out of imprinting on a specific stripe phenotype. Future work should address the effect of the specific form of the stripe patterns: are individuals attending to the salient spatial frequency and orientation characteristics of

the stripes, or more generally to the darker colour of individuals expressing pigment patterns?

Variation in preference for stripes may provide an interesting paradigm for studying the developmental genetics of higher-level visual processing in zebrafish. Video playback may constitute a useful approach for studying visual perception in *Danio*. The procedure used to automate the behavioural assay in the present study is straightforward. Video stimuli can be presented in parallel to large numbers of zebrafish, and association time can be easily quantified using image-analysis software. These properties make video a viable technique for mutagenesis screens. Video playback may prove an important tool for studying mechanisms of visual perception and social behaviour in this model system.

Acknowledgments

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