Research report

Eye use during viewing a reflection: Behavioural lateralisation in zebrafish larvae

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Abstract

Development of visual lateralisation was studied in zebrafish larvae of an outbred strain when examining their own reflection in a mirror. There was significant left eye preference at all ages studied. A decrease in left eye use around 14 days, followed by a later increase at 21 days, parallels similar but differently timed shifts in the domestic chick. Age-dependent shifts in the likelihood of control by one or other eye system may be responsible. Larvae tested at 26 days of age with unfamiliar conspecifics of similar age also used the left eye (LE). Larvae of another strain (TupLF) also LE viewing of their reflection, although this was confined to the first or first and second minutes of the test rather than lasting through 10 min. The reflection differs from prior experience of fellows in the timing and character of its movements relative to those of the larva. Specialisation of the left eye system for the assessment of novelty, which is present in zebrafish, just as in many other vertebrates, probably therefore explains left eye use in the mirror test. Reasons why strains might differ in the length of time that they devote to assessment of the reflection are discussed. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Studies of behavioural asymmetries in vertebrates have in many cases revealed lateralisation at the population level [3,10,23,25]. This is particularly true of the ‘mirror test’ for teleost fish, where adults of eight species, including zebrafish, all showed the same preference for left eye use when examining their own reflection [21,22]. At the same time, other tests have revealed differences between species: a study by Bisazza et al. [8] was particularly extensive, finding differences between sixteen species. It was clear in some cases (e.g. [6,7]) that differences between species or individuals were due to motivational differences (e.g. level of social or sexual interest). However, differential selection in the teleost Girardinus falcatus produced linked changes in a wide variety of tests, suggesting reversal of some key aspect of lateralisation [9,24].

Where lateralisation has been intensively studied in a particular species, a similar pattern of allocation of abilities, which is present in at least a majority of individuals, has been found in a range of vertebrates, including teleost fish, birds and mammals (reviews, [2,19,26]).

In non-mammalian vertebrates use of either left or right eye (LE, RE) when viewing particular types of stimuli has revealed differing specialisations of the visual systems that are fed by LE and RE (LES, RES). The LE is used to assess novelty, which must require comparison of current perceptual input with a record of a previously experienced and similar object or class of objects. This is well exemplified in the zebrafish itself, which uses the LE to examine an object or scene that has been seen once before [14]. A judgement of adequate match or a particular degree of novelty must require examination of a wide range of stimulus dimensions. In larval zebrafish (‘fry’) this includes context: a familiar stimulus in a strange place is examined for some time when the LE is in use but not when the RE is used [27]. Other teleosts show LES assessment of novelty. Xenopus sarasmorum uses the LE to examine a transformation of a familiar pattern [20]. Adult Guppies use the left eye for strangers, but the right for familiar fellows [11].

The right eye (RE) is used by zebrafish in visual control of response (VCR), such as occurs during approach to seize a target [13]. Larval zebrafish (‘fry’) show a number of LES and RES
specialisations like those of the adult [27]. These include LE use in the assessment of novelty, and enhanced ability to sustain a motor strategy when RES is in use.

In the tests reported here, eye use was measured from body posture in zebrafish fry, when they viewed their own reflection for the first time ("mirror test") or a group of fry. In both cases, the stimulus object was similar visually in most ways to the fry with which the test individual had lived. The reflection was visually novel in its locomotion (e.g. it moved only when the fry itself moved). The absence of cues in other sensory modalities may also have been important. Strains differed in patterns of viewing, but invariably it was the LE that was used when bias in eye use first appeared. In TupLF and a strain derived from TupLF ("fsi"), the period of LE viewing was brief, as would be expected if assessment of novelty were to cease, once a record of the novel properties of the stimulus had been recorded. However, in one strain ("Outbred"), LE use was surprisingly persistent, lasting to some extent for at least 10 min. Here differences from prior experience continued to affect behaviour despite ample time to establish their precise nature. Imprinting in birds [5,12] provides a possible parallel. Here, once exposure to an appropriate object leads to social attachment, transformations of that object are persistently avoided, even though without prior imprinting they would have been attractive.

In the domestic chick there are sharply tuned changes in the likelihood of LES or RES taking charge which affect a wide range of behaviours. On day 8 it is the RES and on days 10–12 the LES (review: [1,16–18]; Section 5). We present evidence of age-related changes in eye use in zebrafish fry that may be comparable.

Two strains were used. One ("Outbred") was originally derived from a strongly outbred stock held by Dr. S. Wilson (Dept. Anatomy and Development, UCL). The other was the TupLF strain, much used in genetic work, and of special interest to studies of lateralisation since it was the source of the fsi strain used in studies of the effects of diencephalic reversal on lateralisation behaviour [4]. The striking difference in patterns of eye use between the two strains has already been noted [4].

2. Experiment 1

In view of the existence of marked changes in bias to use of LES and RES during development in the domestic chick (Section 2.3), fry were tested at a range of ages.

2.1. Methods

Outbred fry were used. Each experimental group was of 14 fry. Each group was tested once only. Five ages were examined: 8, 12, 14 (two groups) and 21 days of age. Fry were kept in small white plastic tanks (13 × 7.5 cm, with 4–5 cm depth of water), standing in a larger tank, which was maintained at 27°C. A 12/10 cycle of 14:10 h was maintained. A commercial larval diet (ZM 100, Atlantic Aquatics, greta Yarmouth, UK) was fed from day 6 onwards.

The test tank (20 × 5 × 8 cm) had mirrors as the two longer walls, whilst the shorter walls and the floor were white (Fig. 1); the water was 2.5 cm deep. Lighting was from above (60 W bulb). Each fry was placed in turn in the middle of the apparatus and video-recorded from above for 10 min. Fry positions were scored every 2 s by superimposition on the computer screen of a cursor on the long axis of the body, using the video recording. Body angle was taken to relate to the closer mirror. Positions when the fry was in a central strip 4 mm wide (Fig. 1) were discarded. Positions in which fry were aligned parallel with the mirror ("parallel observations"), as judged by eye, and those in which fry were instead at an angle to the mirror ("angled observations") were recorded separately. Significant differences between data for the two body postures occurred, but only in the second experiment, which used 26-day fry, and groups of other larvae as the stimulus, rather than a reflection. Eye use during the first and second 5-min of test was analysed separately, since patterns commonly change over this period of time in tests with adult fowl [21]. This proved to be the case here as well.

An index of eye use was calculated as [(frequency of right eye use)/(frequency of right eye use + frequency of left eye use)] × 100. Values significantly higher than 50% would thus indicate preference for right eye use, and values significantly lower than 50%, preference for left eye use. Significant departures from chance level (50%) were estimated by two-tailed one-sample t-test. Further analyses were carried out by analysis of variance (ANOVA).

2.2. Results

Analysis of angled data (Fig. 2a) showed a significant effect of age (F1,52 = 3.000, p = 0.039). Time (first versus second 5 min: F1,52 = 0.071) and age × time (F3,52 = 0.700) were not significant. Parallel data (Fig. 2b) showed a suggestive effect of age (F1,52 = 2.162, p = 0.104). Neither time (F1,52 = 0.071, p = 0.79) nor age × time (F1,52 = 1.119, p = 0.408) were significant.

When all viewing postures were included (i.e. angled and parallel data were lumped), there was a significant main effect of age (F1,52 = 2.71, p = 0.045), whereas time (first versus second 5 min of test; F1,52 = 0.009, p = 0.923) and the age × time interaction (F1,52 = 0.895, p = 0.45) were not significant. Post hoc analyses (Fisher’s least significant test, p < 0.05) revealed significant differences between days 8 and 14, and between days 14 and 21. The changes between days 8 and 14, and between days 14 and 21 were partly due to changes in eye use over the course of the test. On day 8, left eye use became unusually marked in the second 5 min, whilst on day 21, it was unusually marked in the first, but decreased somewhat in the second 5 min (Fig. 2a and b). On the days in the middle of the time-course, left eye use was moderate and did not change with time. A suggestive time by age interaction resulted, when days 8 and 21 were compared (F1,26 = 3.162, p = 0.087). Despite these changes with age, there was significant bias to left eye use at every age (Fig. 2c).

As a further control we tested a separate group of fry at day 14 of age (N = 14). The results confirmed the previous ones: the replication showed the same results as those of the original experiment (14 days; mean = 42.55, S.E.M. = 2.611; 14 days replication: mean = 40.27, S.E.M. = 2.832; t4 = 0.504
Fig. 2. Results of Experiment 1. Percentages of right hemifield use. Group means (with S.E.M.) are shown. (a) Angled observations; (b) parallel observations; (c) lumping together all observations (asterisks indicate significant departures from chance level, using one-sample two-tailed \( t \)-test).

\( p = 0.558 \). When these fry were compared (data lumped across both time and parallel/angled) with the older ones (21-day-old) of the previous experiment a significant difference was apparent (\( t_{26} = 2.295 \), \( p = 0.03 \)).

2.3. Discussion

The domestic chick shows sharply timed changes during development in the relative likelihood that RES or LES will control behaviour (review: [1]). A suite of changes on days 10–12 indicate increased LES control. Chicks for the first time move independently of the mother hen, and leave her to enter the environment on their own [28]. An earlier point of enhanced RES control on day 8 is revealed by reliance on local cues in a test where distant topographical cues are also available, and used in the later LES period [15]. Both points can be understood as producing changes in behaviour that are advantageous in a species, in which changes in individual members of the brood and in the mother need to be coordinated.

The age-related changes found in the Outbred strain do not appear to represent a comparable condition. Instead, there is clear bias to LE use at all ages. Variation in interest in the properties of other fry is an adequate explanation for the changes over age.

3. Experiment 2

A second type of test was used to see what pattern of viewing might be used to more normal test objects. Older fry (day 26) were used, both as viewers and as a group of test objects. In view of the evidence for less persistent interest in the reflection on day 21, it seemed possible that a new pattern of viewing might be found.

3.1. Methods

Fourteen fry were used at 26 days of age. Seven of them had been used in a mirror test; the other seven were naive. The apparatus consisted of a swimway (20 × 5 × 8 cm), with white walls and floor. At one end of the swimway, a small egg-shaped tank (5 × 2.5 cm), with white walls, was so placed that the fry in the swimway could see into it through one of the smaller ends (Fig. 3). The small tank contained nine unfamiliar fry, also 26 days old. Lighting was from...
above (60 W bulb). The water depth in both tanks was 2.5 cm deep. The floor of the swimway was divided into eight strips, each 2 cm wide, and at distances from 1–2 to 15–16 cm from the group of conspecifics. At the start of the test the fry entered the swimway through an aperture 1 cm wide. Its behaviour was video-recorded from above for 10 min.

Body position was scored every 2 s, together with which strip the fry was then in. Positions in which the body of the fry touched the walls of the swimway were discarded from analysis, since this might have constrained posture. Other procedures were as in Section 2.

3.2. Results

ANOVA's were carried out using time (first 5 versus second 5 min), and distance, considering parallel and angled postures separately.

In the case of parallel postures (Fig. 4a) there was a suggestive interaction between time and distance ($F_{7,84} = 1.821, p = 0.094$). No other effects approached significance (time: $F_{1,12} = 0.267, p = 0.614$; time × experience: $F_{1,12} = 0.257, p = 0.621$; distance: $F_{1,12} = 1.782, p = 0.102$; distance × experience: $F_{1,12} = 1.408, p = 0.213$; time × distance × experience: $F_{3,36} = 1.060, p = 0.396$; experience: $F_{1,12} = 0.000, p = 0.810$). Separate analyses revealed a significant effect of distance during the first 5 min ($F_{7,91} = 3.211, p = 0.004$), but not during the second 5 min ($F_{7,84} = 0.486, p = 0.842$). During the first 5 min (Fig. 4a), there was striking use of the left eye in the 8 and 10 cm strips, which was flanked by distances at which there was instead greater use of the right eye (4 and 6 cm, and 12 cm). Elsewhere there was left eye use. In the second 5 min (Fig. 4a), this pattern largely disappeared, leaving an overall bias to the left eye use ($t_{13} = -3.03, p = 0.010$).

There were no significant effects for angled postures (Fig. 4b; time: $F_{1,12} = 0.0001, p > 0.1$; time × experience: $F_{1,12} = 0.241, p = 0.632$; distance: $F_{3,34} = 1.005, p = 0.434$; distance × experience: $F_{3,34} = 1.256, p = 0.282$; time × distance: $F_{3,34} = 1.340, p = 0.242$; time × distance × experience: $F_{3,34} = 0.421, p = 0.886$; experience: $F_{1,12} = 1.915, p = 0.192$). The left eye was used predominantly (first 5 min: $t_{13} = -6.94, p = 0.0001$; second 5 min: $t_{13} = -2.86, p = 0.013$).

When parallel and angled observations were considered together (Fig. 4c) there was a general and striking preference for left eye use (first 5 min: $t_{13} = -8.19, p = 0.0001$; second 5 min: $t_{13} = -5.80, p = 0.0001$; overall 10 min: $t_{13} = -8.62 p = 0.0001$).

When the time spent at different distances was measured (Fig. 5), it was clear that most time was spent very close to the glass dividing the test fry from the group of fry, with a fall off in duration spent in each strip at greater and greater distances. There was, as a result, a significant effect of distance ($F_{7,92} = 6.022, p = 0.0001$). Time spent viewing was less in the second, than in the first 5 min ($F_{1,13} = 27.764, p = 0.0001$). The interaction distance × time was not significant ($F_{7,91} = 0.554$).

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Fig. 4. Results of Experiment 2. Percentages of right hemifield use. Group means (with S.E.M.) are shown. (a) Parallel observations; (b) angled observations; (c) lumping together all observations.
3.3. Discussion

It is clear that despite the changes in procedure, LE bias was once again obtained.

4. Experiment 3

The TupLF strain was used because it was known [4] that a derived strain (fis) showed only very brief LE bias in mirror tests. Fry were used, reared and tested (on day 8) exactly as were Outbred fry in Experiment 1, except that tests were only 5 min in duration. Two separate batches were tested (replications 1 and 2).

4.1. Methods

Fourteen (first replication) and 20 (second replication) TupLF fry were used, reared and tested (on day 8) exactly as were Outbred fry in Experiment 1, except that tests were only 5 min in duration. The fry proved to show LE use only at the beginning of viewing.

4.2. Results

In the first replication, the main effect of time was not significant ($F_{4,52} = 1.413, p = 0.243$). However, the bias to LE use in the first minute (Fig. 6a) was significant ($t_{13} = -2.207, p = 0.046$), providing a basis for prediction in subsequent replications. In the second replication, there was a significant effect of time ($F_{4,76} = 3.393, p = 0.013$), which was due to the bias to LE use in the first and second minutes (Fig. 6b). This was significant in both minutes ($t_{19} = -2.104, p = 0.049; t_{19} = -2.590, p = 0.018$).

4.3. Discussion

In this strain fry thus begin with a somewhat variable period of LE use, which is no more than 1 or 2 min long. They certainly do not sustain such viewing throughout (at least) the first 5 min, as did Outbred fry.

5. General discussion

The mirror test, which has been so successful in demonstrating population level lateralisation in adult teleosts [20,21], is here shown to do the same for young zebrafish fry. The evidence given here suggests that the widespread use of the LE in such tests is because novel features of the reflection cause general assessment for novelty. The same may be true when viewing not a reflection, but groups of fry (Experiment 2), even though the unusual features of a reflection are here absent. The latter include movement only during movement of the fry itself, which is away when the fry retreats, and towards the fry when it advances. However, lateral line cues, which would normally accompany locomotion of fellows, are absent, and there may also be absence of chemical cues. The assumption of a special distance for LE viewing is best explained by assessment of novelty, accompanied by temporary avoidance [20]. It is clear from the time spent at various distances from the partition that the test fry tended to attempt close approach for much of the time, whereas the LE viewing zone was removed from the partition.
by 8–10 cm. The zone of preferred LE viewing was bounded by zones in which there was significant shift away from LE view-
ing, probably as part of locomotion either towards or away from the group of fry. This in turn may mark decisions to avoid or approach the group, following a period of assessment.

The TupLF strain, in contrast, shows relatively brief initial LE viewing in mirror tests, followed by the rest of the test in which there are no clear periods of bias to either eye use. This is consistent with the usual pattern of behaviour shown by animals when they encounter a novel object: initial assessment of novelty is followed, once completed, by a loss of interest.

It is possible that differences in development underlie the strain difference. Developmental changes occur in the Outbred strain. The delayed development of full LE use at day 8 is consist-
tent with a condition in which a period of facilitated learning of the visual characteristics of other fry is just beginning. By days 12 and 14, interest in the reflection begins at once, as though a detailed record of prior experience of fry comes at once into use. It is sustained throughout the test, whereas by day 21 the bias to LE viewing decreases towards the end of the test. This may owe less to the end of a period of facilitated learning about the appearance of other fry. By day 21, then, differences between prior experience and the properties of the reflection become of less interest within the course of the test.

It remains to be seen whether TupLF begin a period of sus-
tained interest in the properties of other fry later than the Outbred strain or not at all. Genetic variation in this aspect of development may be important in adjusting larval behaviour to selection pressures from predation [10]. Greater or lesser degrees of associa-
tion could balance the disadvantages of being found because an aggregation is found against dilution of risk by the presence of many alternative targets.

References
[2] Andrew RJ. The earliest origins and subsequent evolution of later-
[19] Rogers LJ, Andrew RJ. Comparative vertebrate lateralization. Cam-
[25] Vallortigara G, Rogers LJ. Survival with an asymmetrical brain: advan-
[28] Workman L, Andrew RJ. Simultaneous changes in behaviour and lat-