Further evidence for mirror-reversed laterality in lines of fish selected for leftward or rightward turning when facing a predator model

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Abstract

In the teleost fish *Girardinus falcatus* eye preferences for inspecting a potential predator is highly heritable and this consented to select lines with opposed laterality. In previous studies individuals from a RD line (rightward turning when facing a dummy predator) and those from a LD line (leftward turning) were subjected to several other laterality tests (most of which, possibly all, were visually based). Since they obtained opposite scores in all tests, it was suggested that LD and RD fish have complete mirror-reversed organizations of the brain. Here, we studied fish from selected lines in a new set of laterality tests including some non-visual tests. They included measures of (1) rotational preference in the home tank (2) direction of spontaneous swimming in the dark (3) escape trajectories after delivery of an auditory stimulus (4) escape turning direction to a fast approaching visual stimulus. The results of the first three tests are congruent with the finding of previous studies in that fishes of the two lines showed opposite direction of lateralisation. When tested for laterality in the escape response to a fast approaching stimulus, fish of the LD and RD lines showed no differences and both were biased toward leftward escape. Overall these results suggest the existence in *G. falcatus* of a single mechanism controlling a co-ordinate placement of the great majority of lateralised functions. Yet the results of the fourth test suggest that a few lateralized functions are controlled by different mechanisms that were unaffected by artificial selection.

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1. Introduction

In humans as well as in other vertebrates a large number of cerebral functions appear to be lateralised [26]. Since the 19th century, the left hemisphere in humans is known to be specialised for language. The same side of the brain controls auditory communication in other mammals, in birds, amphibians [1,21,22] and possibly fish [12]. The right side of the brain is specialised for other functions. For example the right hemisphere in primates is activated during face recognition tasks [19,27]; the same side of the brain appears to control social recognition in a number of other species including birds, amphibians and fish [7,28,29].

A consistent finding of studies that have looked in detail at human and animal laterisation, is the presence within a population of individual variation with some individuals showing a reverse lateralisation or bilateral representation of the function. Left-handedness for example is found in around 10% of the individuals with some variation across populations [18]. Ten percent is also the frequency of atypical right hemisphere dominance for language [24]. These early data were obtained mainly from patients with brain lesions but a similar frequency of atypical language laterisation has been confirmed by recent neuroimaging techniques in the normal population [17,23]. A detailed comparison of hemisphere activation between individuals with typical left dominance and individuals with right dominance recently conducted by Knecht et al. [16] suggests that the latter constitute a natural variant of language lateralisation rather than a re-organisation following an early undetected brain damage. Similar varia-
tion naturally occurs in other vertebrate species, although atypical patterns of lateralisation appear more frequently in non-human vertebrate populations, being commonly found at a frequency of 30–35% [2,8,11].

Since the discovery of individual variation in lateralisation, scientists have begun to ask if individuals with atypical lateralisation in one function do show atypical lateralisation in all other functions or alternatively each function lateralise independently. Rasmussen and Milner [24] found a greater frequency of individuals with atypical language lateralisation among left-handers. However the association was not very strong as only 30% of left-handers had atypical language lateralisation. By comparison, in their study atypical language lateralisation was found in 5% of right-handers, but recent data suggest it may indeed be more frequent [17]. More recently Floel et al. [13] measured lateralisation of spatial attention in 10 subjects with typical and 10 with atypical language lateralisation using transcranial Doppler sonography. All subject with typical language lateralisation also showed typical lateralisation of spatial attention (left hemisphere dominance). Six out the ten subjects with atypical language lateralisation showed reversal of lateralisation also for spatial attention; the remaining four subjects showed typical lateralisation of spatial attention and therefore right hemisphere carried out both language and spatial attention functions.

An association among different aspects of lateralisation have been observed in other studies but the correlation found is never very high [15,25].

A recent paper on laterality of cooperative predator inspection in Gambusia holbrooki showed that in a natural population nearly 75% of the fish approached a predator by keeping the stimulus on their left eye and fixating it with the right eye when in its vicinity, whereas the remaining 25% of the fish did the opposite [8]. A more comprehensive study was done in a closely related species, Girardinus falcatus using fish from lines selected for opposite lateralisation. Selective breeding was based on a detour test that measured the direction taken by a fish when it met a dummy predator partly visible behind a barrier [30]. Fish of selected lines for right turning (RD fish) and for left turning (LD fish) were subjected to the same battery of laterality tests including some non-visual tests. Most tests did in fact measure eye preference as detour tests do [11]. The two apparently non-visual tests (rotation in a circular arena and T-maze turning) may also have an important visual component. In G. holbrooki, clockwise/anti-clockwise direction of swimming was shown to be strongly affected by the nature of an object placed in the middle of the arena [2] and it is possible that even in absence of a precise object, fish might be influenced in their turning direction by their preference for visually explore the new environment with one eye. Bisazza et al. [3], used a very similar test to the T-maze used here and suggested that turning preferences might be the result of the tendency to use one particular eye to look ‘around the corner’ for potentially important stimuli rather than merely reflecting the presence of motor biases.

In this study we have tried to unravel this problem by presenting G. falcatus of the RD and LD lines with a wider range of laterality tests including some non-visual tests.

2. Materials and methods

2.1. Subjects

Fish used in this study were males and females of G. falcatus (Teleostei, Poeciliidae). We compared two groups of fish with opposite laterality in the detour test. The procedure of the detour test has been described in detail elsewhere [4]. Briefly, the apparatus consisted of a large tank with a swimway in the middle allowing the fish inside to face, at the end of the swimway, a vertical-bar barrier behind which the visual stimulus was located. The visual stimulus consisted in a model of predator. The fish was gently pushed using a pair of fish nets at the starting point of the runway. He swam along the runway until it faced the barrier. Ten consecutive trials were given and we computed the percentage of right and left turn taken by the fish when leaving the runway.

Fish that turned 80% or more leftward in the detour test were labelled as left detour (LD); fish turned 80% or more rightward in the detour test were labelled as right detour (RD).
LD and RD fish derived from generations five, six and seven of four lines selected for laterality in the detour test. Two lines were selected for rightward turning and two for leftward turning. For the experiments we used adult fishes after they had produced their second litter (approximately 6-8-month-old). Individuals were maintained in small heterosexual groups (10-15 fish) of the same laterality, kept in 70-l glass aquaria with abundant vegetation (Ceratophillum sp.) and artificial lighting 16 h a day; water temperature was maintained at 25 ± 2 °C and all fish were fed dry fish food and Artemia salina naupli twice a day.

Individuals were never used twice in the same test but it is possible the same fish may have been used in more than one experiment. In the latter case, at least one month occurred between the two experiments. Statistical analyses were done with SPSS statistical Package. We use Student T-test for unequal variances whenever homogeneity of variance was not met.

2.1.1. Rotational preference in a familiar environment
Turning preferences in a novel environment may be driven by the preference to look at the novel scene with a given eye [2]. The aim of this test was to measure turning preference after the subjects were thoroughly acquainted with the environment, so to minimize the possible influence of eye preference for looking at novel stimuli.

Fourteen LD females and fourteen RD females were used for this experiment. Each fish was individually housed in a tank consisting of a ring-shaped swimway (inner radius: 12.7 cm, outer radius 28 cm, height 27 cm) made of white plastic material with 2 cm of gravel substrate and filled with 15 cm of water. Each tank was covered with a thick grey plastic net acting as one-way screen and preventing the fish from seeing any object outside his test tank. Swimming behaviour of the fish was recorded by a video camera mounted 2 m above the tanks. Each camera recorded four adjacent tanks (two with a RD and two with a LD fish).

Each subject was dip-netted in its aquarium, transported to one experimental tank and let in it undisturbed for 72 h. Fish were fed dry fish food once a day. The fourth day the behaviour of each fish was video recorded for 4 h (2 h in the morning and 2 h in the afternoon). Lateral asymmetries were analysed from video recordings by computing for each subject the percentage of clockwise rotation.

2.1.2. Turning direction in the dark
After a sudden turning off of the light, fish continue to swim for some minutes. In this experiment we measured turning directions during swimming in the dark when visual references were absent.

Ten LD females and ten RD females were used for this experiment. The apparatus consisted of a plastic circular tank (upper diameter 102 cm, lower diameter 98 cm, 36 cm high) placed in a completely darkened room. An infra-red video camera was mounted 160 cm above the apparatus in order to video record the fish in absence of visible light. The tank was filled with 11 cm of water and lit with four 20 W infrared lamp arranged on a square-shaped loom placed 120 cm above the tank. Each subject was dip-netted in its aquarium and transported into an adjacent darkened room where it was introduced into a hollow cylinder (diameter 10 cm) in the centre of the experimental tank. After 10 min the cylinder was carefully raised up and recording begun. Every trial was video recorded for 30 min while the experimenter could see the session on a monitor outside the darkened room. During normal swim, turning consists of a clear change of direction induced by caudal fin toward left or right. Video recordings were analysed frame by frame (video recorder SONY SLV-SE40); only swim behaviours that occurred 10 cm from the wall and changes of direction that were of at least 10° respect to the previous direction were considered. We considered the first 10 change of direction for each fish. Lateral asymmetries were calculated for every subject as percentage of right turn.

2.1.3. Escape trajectories elicited by auditory stimuli
C-start escape responses are triggered by a variety of stimuli. They are mediated by a simple neural circuit and are characterized by an extreme promptness. A C-start is a ballistic response and once triggered it does not rely on any other sensory information to be brought to completion. In this test we measured the direction of a C-start escape response triggered by an auditory stimulus.

Twenty-one LD females and eighteen RD females were used for this experiment. The apparatus (Fig. 1) consisted of a circular opaque plastic tank (upper diameter 54 cm, lower diameter 43 cm × 25 cm high) located on a hollow cylinder (diameter 45 cm). The acoustic stimulus was produced by a speaker (50 W) located on the lower part of the cylinder facing the tank at a distance of 16 cm. The apparatus was filled with 8 cm of water and lit by a fluorescent circular lamp (70W) placed externally the apparatus. The acoustic stimulus consisted of a single tone (50 Hz, 0.5 s) generated by a computer.

![Fig. 1. Schematic representation of the apparatus used in the second experiment.](image)
A small LED (which could not be seen by the fish) was on when the speaker was active. A video camera was mounted 130 cm above the apparatus; the monitor, video recorder and computer were located outside the experimental room. Each subject was dip-netted in its aquarium, transported into the experimental tank and kept in there for 30 min to become accustomed to the novel environment. In order to avoid artefacts, the stimulus was presented only when the subject was in the centre of the tank. Stimulus presentation was repeated 10 times with a minimum inter-trial interval of 10 min. Video recordings were digitised and analysed on a computer frame by frame. Because the response to the stimulus was a typical C-start such as occurs in less than 40 ms from stimulation [9] we analysed separately the odd field and even field of each frame thus allowing a resolution of 50 frames per second (a 20 ms interval). We recorded the direction of the first lateral body contraction and we measured, at the onset of stimuli, the distance between the subject and the centre of the tank, as well as the angle created by the antero-posterior axis with the vector radius from the fish head during the stimulation.

2.1.4. Escape turning direction to a fast approaching visual stimulus

In this experiment we replicated the test used by Cantalupo et al. [6] to measure laterality of escape response to a fast approaching visual stimulus in an unselected population of G. falcatus. The rationale for this test is that fast responses (C-starts) to a visual input are mediated by a reticulospinal circuit and not by the tectal and prosencephalic systems start to a visual input are mediated by a reticulospinal circuit close to the visual stimulus in an unselected population of teleosts. In this experiment we replicated the test used by Cantalupo et al. [6] to measure laterality of escape response to a fast approaching visual stimulus in an unselected population of G. falcatus. The rationale for this test is that fast responses (C-starts) to a visual input are mediated by a reticulospinal circuit and not by the tectal and prosencephalic systems start to a visual input are mediated by a reticulospinal circuit.

Subject used for this experiment were 72 (37 LD 35 RD) females. An additional small group of males (16 LD and 16 RD) was tested to check for possible gender differences. The apparatus was similar to that used in a previous experiment [6]. It consisted of a glass aquarium (60 × 19 × 35 cm) internally covered by white plastic material except for the front transparent (through which the stimulus was presented) and the upper side closed by a one-way screen. The stimulus consisted of a white oval “predator shape”, with two little black disks (eyes; diameter 10 mm) arranged horizontally on the upper part of the shape, and a black horizontal bar (“mouth”; 3 mm × 35 mm) on the lower part of the shape. The stimulus was fixed on the lower end of a black plexiglass stick (65 × 4.5 cm) which was in turn pivoted on a stud. The stick with the stimulus was raised by hand and was kept in position by an electro-magnetic device; it could then be released by means of a remote electric control. In the end part of the aquarium we placed a green plastic barrier consisting of a series of bars aligned vertically and spaced 0.5 cm apart in order to provide a safe place for the fish to hide after the stimulation. To carry out simultaneously more trials, six identical tanks were located one close the other. Every aquarium was filled with 27 cm of water, illuminated by a fluorescent lamp (58 W) and had a 2 cm gravel substrate. A video camera was mounted 140 cm above the apparatus.

Each subject was placed in a tank the day before the test and let to settle overnight. During the test the experimenter was approximately 1.5 m from the tank sitting in the dark. Twenty escape-reactions for each subject were recorded. There was a minimum interval of 5 min between stimulus presentations. The stimulus was presented as the fish swam across the anterior portion of the tank (at a distance from the front glass comprise between 10 and 28 cm), being in a central position (in relation to left and right sides of the tank) and having its central axis perpendicular to the side through which the stimulus appeared. These conditions were later checked on the videotape and those presentations not fulfilling these requirements were omitted from the analysis. Statistical analysis was conducted on the percentage of right turning.

3. Results

3.1. Rotational preference in a familiar environment

There was no significant difference in activity (total number of rotations) between the two lines of fish in the morning (t(26) = 1.522; NS) or in the afternoon (T-test for unequal variances, t(17,337) = 2.05; NS). RD fish swam preferentially anticlockwise while LD fish swam preferentially clockwise (Fig. 2). The difference between the two groups is significant (t(26) = 2.175; P = 0.039). A two-way analysis of variance confirmed a significant difference between RD and LD fish (F(1,26) = 5.831; P = 0.023) but revealed no morning/afternoon difference (F(1,26) = 0.246; NS) or interaction (F(1,26) = 0.403; NS). Neither RD nor LD lines showed a significant departure from chance level although the latter was marginally non significant (RD, t(13) = 1.06; NS; LD, t(13) = 2.0; P = 0.067).

3.2. Turning direction in the dark

RD fish made more left turns and LD fish made more right turns (Fig. 2). The difference between the two groups was significant (T-test for unequal variances t(12,344) = 2.496; P = 0.027). LD but not RD lines showed significant departure from chance level (RD, t(17) = 1.34; NS; LD, t(20) = 2.88; P < 0.02).

3.3. Escape trajectories elicited by auditory stimuli

In approximately 90% of the cases a C-start was observed in the semi-field of a frame successive to the onset of stimulus. In the remaining cases the C-start was observed in the following semi-field. Therefore, all C-starts had a latency ranging approximately from 20 to 40 ms, which is the expected latency for a escape response mediated by Mauthner cells.

LD and RD fish lines differed in the preferred direction of contraction. LD fish contracted more often to the left while...
RD fish contracted more often to the right (Fig. 2). Differences between RD and LD fish lines was significant (T-test for unequal variances $t_{(35.358)} = 2.103, P = 0.043$). RD but not LD lines show significant departure from chance level (RD, $t_{(17)} = 2.14, P = 0.05$; LD, $t_{(20)} = 0.75$, NS).

At the time of stimulus’ presentation RD fish were displaced on average $0.33 \pm 8.86$ cm from the geometric centre with the right side of the body facing the centre of the apparatus, while LD fish were on average $1.64 \pm 9.19$ cm from the centre with the left side of the body facing the centre. This difference between RD and LD fish was not statistically significant (repeated measure analysis of variance $F_{(1,34)} = 2.13, P = 0.159$). However, because the variance was high, the degree of displacement from the centre could have influenced the reaction of the fish. To check this hypothesis a logistic regression was carried out. The dependent variable was the direction of the contraction and the predictor variables were the distance from the centre of the apparatus and the angle created by the antero-posterior axis and the vector radius from the fish head. None of these measures was found to influence escape direction (logistic regression: $c_2(2) = 1.57$, NS; distance from the centre: $B = 0.0069$, Wald = 0.33, NS; angle: $B = 0.0052$, Wald = 1.24, NS).

A comparison of the results of this experiment and the previous one indicates that the direction of spontaneous slow turns was reversed in the fast turns elicited by an auditory stimulus. The difference is significant in both LD fish ($t_{(26)} = 2.46, P < 0.05$) and RD fish ($t_{(28)} = 2.49, P < 0.05$) though the sign of the difference was opposite in the two groups.

### 3.4. Escape turning direction to a fast approaching visual stimulus

Frame by frame analysis showed that escape responses in this test were different from those elicited by the auditory stimulus in the previous experiment. They appeared to be considerably slower and in most cases two to three full frames (80–120 ms) were required for a complete turn of the fish. Turns were usually very precise and in very few instances did the fish collide with the wall, which suggests the presence of a close sensory control during escape response.

No significant difference was found in this test between LD and RD fish ($t_{(30)} = 0.48$, NS) Overall analysis (RD and LD together) revealed a population bias to escape to the left (mean $\pm$ S.D.: 43.6 $\pm$ 22.2; $t_{(11)} = 2.45, P = 0.007$) (Fig. 2). To check for a possible sex effect, we compared our sample with a sample of males. Analysis of variance revealed no sex differences ($F_{(1,83)} = 0.672$, NS), no differences between LD and RD fish ($F_{(1,83)} = 0.047$, NS), and no interaction ($F_{(1,26)} = 0.039$, NS). Males and females showed an overall bias to escape leftward (mean $\pm$ S.D.: 43.7 $\pm$ 23.3; $t_{(87)} = 2.53, P = 0.013$).

### 4. Discussion

In previous studies we found that fish of LD lines, artificially selected to turn left in front of a potential predator (and hence inspect it with their right eye) showed a significant bias in several other laterality tests too. In particular, the same fish preferentially shouled keeping a companion visible on their right side, swam clockwise when placed in a new circular arena and turn left in a T-maze test. Males approached a prospective female mainly keeping the stimulus on their left side and looked at rivals with right eye prior to an attack.

Fish of RD lines, selected to turn right in front of a potential predator (and hence inspect it with their left eye) showed significant bias in the opposite direction in all tests [5,11,30]. In the present study, fish of LD and RD lines showed significant differences of laterality in three out of four experiments. LD
fish accustomed to a ring-shaped home-tank swim significantly clockwise (corresponding to making left turns) while RD showed a tendency to swim anti-clockwise. When observed to move freely in the dark, LD turned significantly more to the right, while they showed a mild tendency to turn to the left when escape behaviour was elicited by an auditory stimulus. Conversely RD fish tended to turn left in the dark while they turned significantly to the right in response to the auditory stimulus.

The results of these tests thus confirm and extend those of previous studies. They corroborate the idea of an almost completely mirror-reversed organization of the brain in fish of lines selected for opposite laterality in the detour test.

The hypothesis, based on the results of previous studies, that artificial selection may have acted on a trait exerting a general control on visual lateralisation should be reconsidered in the light of the new results of the present study. Turning direction in the dark clearly could not depend on visual stimuli. It is probably a motor asymmetry although we cannot exclude in principle the implication of other sensory modalities such as the lateral line system. In the third experiment we measured a fast escape response to an auditory stimulus. Fast escape response (usually called C-start) is suggested that at least another genetic mechanism exists, which independently controls laterality of escape response to a fast approaching visual stimulus. Nonetheless the concept that mechanisms that govern placement of the different functions in humans and fish are different is reinforced by the hypothesis previously proposed of the existence in G. falkatus of a single developmental mechanism controlling a coordinate placement of the great majority of lateralised functions, including many functions not directly related to visual lateralisation. In addition to this previously known finding it is suggested that at least another genetic mechanism exists, which independently controls laterality of escape response to a fast approaching visual stimulus. Nonetheless the concept that mechanisms that govern placement of the different functions in humans and fish are different is reinforced by the present data. However, as we noticed earlier [5] in humans there are no equivalents of artificially selected lines for direction of lateralisation and therefore it would be crucial to verify if complete mirror-reversed organizations of the brain characterize also the individuals in natural, unselected populations of fish.

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