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SHORT COMMUNICATION

Severe hypoxia impairs lateralization in a marine teleost fish

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ABSTRACT

In intertidal environments, the recurring hypoxic condition at low tide is one of the main factors affecting fish behaviour, causing broad effects on ecological interactions. We assessed the effects of hypoxia on lateralization (e.g. the tendency to turn left or right), a behaviour related to brain functional asymmetry, which is thought to play a key role in several life history aspects of fish. Using staghorn sculpin (*Leptocottus armatus*), a benthic fish that typically inhabits the intertidal zone, we found that hypoxia affects behavioural lateralization at the population level. On average, staghorn sculpins showed a distinct preference for right turns under normoxic conditions (>90% oxygen saturation), but an equal probability of turning right or left after exposure to hypoxia for 2 h (20% oxygen saturation). The specific turning preference observed in the staghorn sculpin control population is likely to have an adaptive value, for example in predator–prey interactions by enhancing attack success or survival from predatory attacks. Therefore the alteration of lateralization expressed by staghorn sculpins under hypoxic conditions may have far-reaching implications for species ecology and trophic interactions. Moreover, our work raises the need to study this effect in other species, in which a hypoxia-driven disruption of lateralization could affect a wider range of behaviours, such as social interactions and schooling.

KEY WORDS: Hypoxia, Lateralization, Detour test, Staghorn sculpin

INTRODUCTION

Oxygen availability is one of the main factors affecting fish physiology, behaviour and ecology. In marine intertidal environments, fish are exposed to hypoxia events daily, mainly because of tidal rhythms. Moreover, the natural occurrence of hypoxic events is exacerbated by human activities, such as agriculture and discharge of raw sewage (Diaz and Rosenberg, 2008; Richards et al., 2009). The effects of hypoxia on fish biology have been intensively studied, showing numerous interferences such as impaired performance in reproductive output, growth, swimming and schooling (Domenici et al., 2007; Richards et al., 2009).

The brain oxygen requirements are among the highest of all the organs, thus brain sensitivity to hypoxia is likely to be high. Indeed,

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near-lethal hypoxic conditions are known to affect brain physiology (e.g. Nilsson, 1990; Nilsson et al., 1993). Furthermore, mormyrid fish, characterized by a huge brain oxygen uptake, are known to show reduced brain size in chronic hypoxic habitats at both the inter- and intra-specific level (Chapman and Hulen, 2001). By contrast, the impact of hypoxia on behaviours directly related to brain function in fish remains relatively unexplored, although arguably, knowledge of such effects could be of great ecological relevance.

In order to assess alterations of brain functioning in hypoxic conditions in fish, we focused on behavioural lateralization, an expression of brain functional asymmetry that reflects a differential specialization of the two hemispheres in processing information (Bisazza and Brown, 2011). In fish, even though the exact evolutionary processes involved are still not clear, the lateralization patterns observed in a population are thought to reflect selective pressures and to be important for the survival of individuals in many ecological settings such as feeding, predator escape, schooling, social and sexual interactions (e.g. Bisazza et al., 1997; Bisazza et al., 2000; Dadda et al., 2010; Bisazza and Brown, 2011). Although in some cases hemispheric specialization could represent an added cost in information processing (Bisazza and Brown, 2011), the vast of majority of evidence suggests that it confers several advantages. A lateralized brain allows a separation of task achievements: each hemisphere can develop higher specialization in processing specific information, resulting in greater cognitive performance. For example, highly lateralized individual fish (i.e. those with greater individual-level lateralization) outperform non-lateralized ones in spatial reorientation tasks or in simultaneous activities, such as foraging and predator vigilance (Sovrano et al., 2005; Dadda and Bisazza, 2006). Moreover, lateralization is also associated with an enhanced ability to escape from a predator: strongly lateralized fish have a faster reaction time, higher turning rates and longer distance travelled in escape responses (Dadda et al., 2010). In some cases, a bias for a specific directionality of lateralization is observed at the population level: a conspicuous proportion of individuals in a population is lateralized in the same direction (Bisazza and Brown, 2011). This bias appears to be relevant in social species to achieve greater coordination in movements (Bisazza et al., 2000), whereas in non-gregarious species, it could be involved in prey–predator interactions (Yasugi and Hori, 2012).

In this experiment, we assessed the effect of hypoxia on behavioural lateralization in the staghorn sculpin (*Leptocottus armatus* Girard 1854), a benthic fish that typically inhabits the intertidal environment and may therefore experience low oxygen availability (<20% oxygen saturation) during stranding in mudflats and tidepools (Palsson et al., 2008). We exposed two groups of staghorn sculpins to normoxia (>90% oxygen saturation) and non-lethal levels of hypoxia that naturally occur in the sampling area (20% oxygen saturation) (Palsson et al., 2008; Speers-Roesch et al., 2013), respectively, and we observed the subjects in a turning preference test (detour test) to evaluate their behavioural lateralization. We then compared the population- and individual-
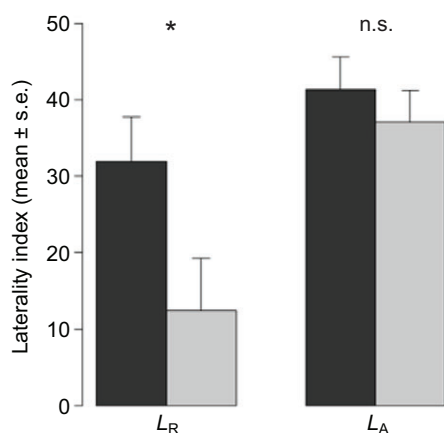
level lateralization of the two experimental groups using the relative laterality index (L_R) and the absolute laterality index (L_A), respectively. The mean L_R allows us to assess the turning bias for a specific direction at the population-level: positive values of L_R indicate a right-turning bias, whereas negative values of L_R indicate a left-turning bias (Bisazza et al., 1997; Dadda et al., 2010). The mean L_A refers to individual-level lateralization and describes the strength of lateralization irrespective of direction (Bisazza et al., 1997; Dadda et al., 2010). Based on previous work that tested the effects of environmental stressors on lateralization (e.g. Nilsson et al., 2012), we hypothesized that exposure to hypoxia may induce a reduction of the individual-level lateralization and/or the population-level lateralization bias.

RESULTS AND DISCUSSION

Individuals were subjected to hypoxia and control water in small groups (see the Materials and methods). Grouping had no significant effect on lateralization pattern (L_R: $F_{10,73}=1.278$, $P=0.259$; L_A: $F_{10,73}=0.725$, $P=0.698$). There was a significant difference in the population-level lateralization (L_R index) of staghorn sculpins groups exposed to normoxia and hypoxia ($t_{82}=2.259$, $P=0.027$) (Fig. 1). On average, control subjects exhibited a right-biased lateralization at the population level, revealed by a distinct preference for right turns when facing the barrier in the detour test under normoxic conditions; the L_R index (31.91 ± 5.87, mean ± s.e.) was significantly greater than zero ($t_{41}=5.413$, $P<0.001$) (Fig. 2). In contrast, the group exposed to hypoxia showed no lateralization bias at the population level (mean L_R index: 12.38 ± 6.85; $t_{41}=1.767$, $P=0.085$). This indicates a reduction of the average turning preference towards the right and an increase in the frequency of individuals with left-turning bias (Fig. 2). The L_A index of the hypoxia-treated group was not significantly different from the control group (hypoxia: 37.14 ± 4.12; control: 41.43 ± 4.17; $t_{82}=0.876$, $P=0.384$) showing there was no effect of hypoxia on the individual-level lateralization (Fig. 1). Because behavioural lateralization is an expression of brain asymmetry, the loss of the turning preference towards the right under hypoxic conditions is likely to be caused by changes in brain hemisphere functioning or neurotransmitter functions, and could have direct implications for the ecology and fitness of individuals in the wild.
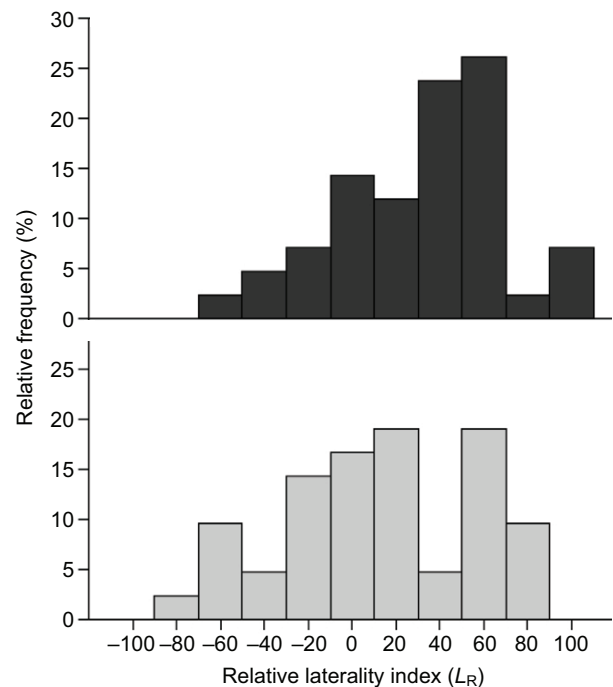
Fig. 2. Response of staghorn sculpin (*Leptocottus armatus*) to hypoxia. Relative frequency distribution of relative laterality index (L_R) in fish exposed to normal levels of oyxgen (black bars) or hypoxia (grey bars). N=42 for control and N=42 for hypoxia.

A shift in information processing between brain hemispheres could explain the effect of hypoxia exposure on population-level lateralization. In several species, the left hemisphere controls behaviours in non-stressful situations, whereas the right hemisphere controls emergency responses (Rogers, 2010). Some evidence and theoretical work suggests that stressful situations could cause a shift of information processing between the two hemispheres (i.e. from left to right control) (Rogers, 2010; Domenici et al., 2014). In line with Rogers (Rogers, 2010), it is possible that the stress due to hypoxia caused treated subjects to increase right-hemisphere control. Using our detour test, an increase in the control by the right hemisphere would imply more frequent turns to the left (Bisazza et al., 1997), resulting in a loss of the original population right-turning bias. In fish, a large within-species variation in coping with a stress situation has been reported. For example, different individuals of juvenile sea bass differentially alter the risk-taking behaviour in hypoxic conditions, probably because of individual variation in sensitivity to hypoxia (Killen et al., 2012). Therefore, it may be relevant to determine whether the effects of hypoxia on behavioural lateralization are more intense in individuals with low tolerance to hypoxia.

The effect on the population-level lateralization may also be caused by a physiological change in neural transmitter functions within the brain following hypoxia exposure. Such changes have previously been reported for CO_2 exposure: behavioural lateralization of coral reef fish larvae maintained in high CO_2 concentration is impaired (Domenici et al., 2012) and the effect is thought to be mediated by changes in brain physiology that alter neuronal transmitter functions (Nilsson et al., 2012). Extreme hypoxia exposure triggers several physiological changes in the fish brain: most fish species rapidly lose ion homeostasis in their brain resulting in a breakdown of brain functioning (Nilsson et al., 1993), whereas others show brain protection against hypoxia as an increase

Fig. 1. Population-level lateralization of staghorn sculpin (*Leptocottus armatus*) is lost under hypoxia. Relative laterality index (L_R) and absolute laterality index (L_A) of fish exposed to normoxia (black bars) or hypoxia (grey bars). N=42 for control and N=42 for hypoxia. Results are means ± s.e. *$P<0.05$; n.s., not significant.

or a redistribution of inhibitory neurotransmitters that suppress brain functioning and limit oxygen requirements (Nilsson, 1990). Similar physiological changes in the brain could explain the loss of the population-level right-turning bias observed in staghorn sculpin exposed to hypoxia. Because species adapted to different environments could show different sensitivities to hypoxic stress, one prospective field of investigation is the between-species variation of the reported effect.

Fish communities in our sampling area may daily experience hypoxic conditions similar to the one recreated in our experiment or even more severe (<20% oxygen saturation) (Palsson et al., 2008), with an increasing occurrence/intensity because of pollution caused by humans (Diaz and Rosenberg, 2008; Richards et al., 2009). As a consequence, the loss of population-level right-turning bias observed in hypoxia-treated staghorn sculpins might have critical ecological repercussions for the species itself, as well as other species. Little is known regarding the ecological meaning of population-level bias in lateralization; however, because the individuals observed in our study were wild-caught fish, their population-level lateralization patterns are likely to have an adaptive value in their natural environment. For instance, in some fish species, escaping predator and attacking prey show lateralized behaviours and the outcome of prey–predator interactions is related to the specific directionality of lateralization (e.g. the preference for approach the prey from right or left) of the two individuals involved (Yasugi and Hori, 2012). In a similar fashion, the lateralization pattern of the staghorn sculpin population used in this study (i.e. most of the individuals had a right turning preference in the detour test) may be adapted to match their prey lateralization patterns in order to enhance attack success, or alternatively to survive from predatory attacks (Vallortigara and Rogers, 2005). The difference between control and hypoxia-treated subjects in population-level lateralization suggests that some of the individuals exposed to hypoxia shift their turning preference from right-biased to left-biased. Therefore, in hypoxic conditions, staghorn sculpins could suffer the severe fitness costs of a decrease in predation success or in survival from predator attacks.

Additionally, in gregarious fish species, population-level bias in lateralization gives advantages for coordination during schooling (Bisazza and Brown, 2011). Staghorn sculpins are solitary and thus do not school. However, similar effects of hypoxia on population-level lateralization may subsist in other fish species as well, including those involved in social behaviours, possibly affecting their schooling activity. Therefore, it may be worthwhile to test the effect of hypoxia on lateralization in gregarious species.

In our experiment, individual-level lateralization was not affected by hypoxia exposure. Inspection of the frequency distribution plots (Fig. 2) offers a possible interpretation for the finding that hypoxia has an effect on lateralization at the population level but not at the individual level. In normoxia, individual sculpins are mainly either non-lateralized or right-lateralized, whereas hypoxia exposure results in some individuals being left-biased. Hence, exposure to hypoxia might cause right-lateralized individuals to become left-lateralized (see peak at LR=−60), resulting in the loss of the original side bias in the whole population while maintaining a similar individual-level lateralization as in normoxia. A similar effect was found in a coral reef fish, where exposure to elevated CO_2 levels affected population-level but not individual-level lateralization (Domenici et al., 2014). In contrast, studies on other species reported an effect of exposure to elevated CO_2 in individual-level but not in population-level lateralization (e.g. Nilsson et al., 2012). At the current stage of research, it is not clear what determines this incongruence of the results. One possibility is that the different

stressors affect lateralization only at the individual or population level in each species. However, one should also consider the possibility that the environmental stressors simultaneously affect both individual- and population-level lateralization, but differences in the effect sizes limit the statistical inference.

Our results show that hypoxia can disrupt lateralization in fish and suggest broad implications for wild populations, because hypoxia is found across most of the world's aquatic ecosystems, and hypoxic areas are spreading worldwide because of increased eutrophication, sediment loading and pollution from human activities (Richards et al., 2009). Moreover, in coastal areas very often fish are simultaneously exposed to other stressors in addition to hypoxia, such as increased temperature, elevated CO_2 or ammonia. Some of these stressors are known to affect lateralization (temperature and elevated CO_2) (Domenici et al., 2014), hence they could potentially interact with hypoxia to alter behavioural lateralization. Therefore, broader investigations on the synergistic effect of different environmental factors on a wide range of species would be of great value to understand the underlying physiological processes and ecological implications of the increasing worldwide incidence of hypoxic zones.

MATERIALS AND METHODS
Experimental subjects
No physical invasive manipulation was performed on the fish. All animal care and experimental protocols followed the guidelines of Institutional Animal Care and Use Committee at the University of Washington (IACUC permit number 4208-3). Eighty-four staghorn sculpins were used overall (standard length 123.54±2.91 mm, mean ± s.e.). Forty-two subjects were exposed to hypoxia, and forty-two were used as control. Each subject was tested only once. Staghorn sculpins were sampled by beach seining at the Jackson beach (48°31′11″N, 123°00′40″W) on San Juan Island (WA, USA) in August 2013. Before the experiments, fish were maintained at Friday Harbor Laboratories (University of Washington) for 24 hours. Maintenance tanks (130×60×26 cm) were kept at a constant seawater flow, under natural light conditions and at a temperature of 11.64±0.52°C.

Experimental treatment
Subjects were transferred in small groups (7–8 individuals) to a treatment tank (45×50 cm) filled with 25 cm of seawater (supplementary material Fig. S1). Water temperature was maintained near constant during the treatment (11.48±0.06°C) in order to avoid any confounding effect of temperature on the oxygen consumption of the subjects. To avoid aerial respiration of the subjects, the water surface was covered with a panel made of transparent synthetic glass. The treatment tank was connected to an external sump (40 cm in diameter, 60 cm high) filled with 50 cm of seawater which allowed constant recirculation of the water into the system. In the bottom of the sump, five standard air stones were installed, one connected to an air pump and four connected to a nitrogen tank providing nitrogen to the sump. A galvanic oxygen probe (Oxyguard Handy, Oxyguard International, Birkeroed, Denmark) connected to an oxygen regulator (PR 5714, PR Electronics, Roende, Denmark) and a solenoid valve controlled nitrogen flow through the air stones, allowing regulation of oxygen saturation in the sump, and indirectly in the treatment tank. For the hypoxia group, we decreased oxygen saturation in the treatment tank from normoxia (>90% oxygen saturation) to 20% oxygen saturation over a 20 min period and kept oxygen saturation constant for 120 min (20.29±0.74% oxygen saturation). For the control group, oxygen saturation was kept at normoxia (98.34±1.02% oxygen saturation) for 140 min and air instead of nitrogen was injected in the sump via the air stones.

Lateralization assessment
Immediately after the end of the treatment (normoxia or hypoxia), subjects were tested for their behavioural lateralization using a detour test (Bisazza et al., 1997), a procedure previously adopted to study lateralization in benthic fishes (Bisazza et al., 2000). The apparatus consisted of a tank (120×50 cm,

water depth 15 cm) with a central runaway (75×10 cm) (supplementary material Fig. S2). At both ends of the runway (6 cm ahead of the runway), a white opaque barrier (20×20 cm) was positioned perpendicular to the orientation of the runway. At the start of each trial, a single fish chosen at random was introduced into the experimental arena and left for 1 min to become accustomed to the environment. Subsequently, by using a pair of small fish nets, the subject was gently conducted up to half of the runway, in order to encourage it to swim toward the barrier. For each subject the procedure was repeated ten times alternating between the two symmetrical ends of the runway (Domenici et al., 2014). The direction of turns in front of the barrier was recorded.

For each subject, we computed the L_R and the L_A (Bisazza et al., 1997). L_R was computed using the formula: $L_R=[(\text{turns to the right}-\text{turns to the left})/(\text{turns to the right}+\text{turns to the left})]*100$, with values ranging from −100 to +100, indicating complete preference for left and right turning, respectively. L_A, ranging from 0 to 100, was computed according the formula: $L_A=|L_R|$ (Bisazza et al., 1997).

Statistical analysis

Because L_R and L_A indices are percentage data, we performed arcsine square root transformation before analysis (Sokal and Rohlf, 1995). To deal with the negative L_R values, we transformed the absolute value of this variable and subsequently we added the corresponding sign to each observation. In the main text, untransformed data are reported for consistency with previous literature. Because the subjects were exposed to hypoxia or control water in small groups, we conducted a preliminary analysis to test whether the group had a significant effect on the L_R and L_A indices using a one-way ANOVA. A mean L_R near zero indicates that a given sample of the population is neither left- nor right-biased in its turning tendency (Bisazza et al., 1997). Therefore, the mean L_R of each group was compared with a theoretical zero using a one-sample t-test (Bisazza et al., 1997). We used the independent sample t-test to compare means of L_R and L_A indices between the control group and hypoxia-treated group. All analyses were carried out using R statistical software (version 3.0.2, R Foundation for Statistical Computing).

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Supplementary materials
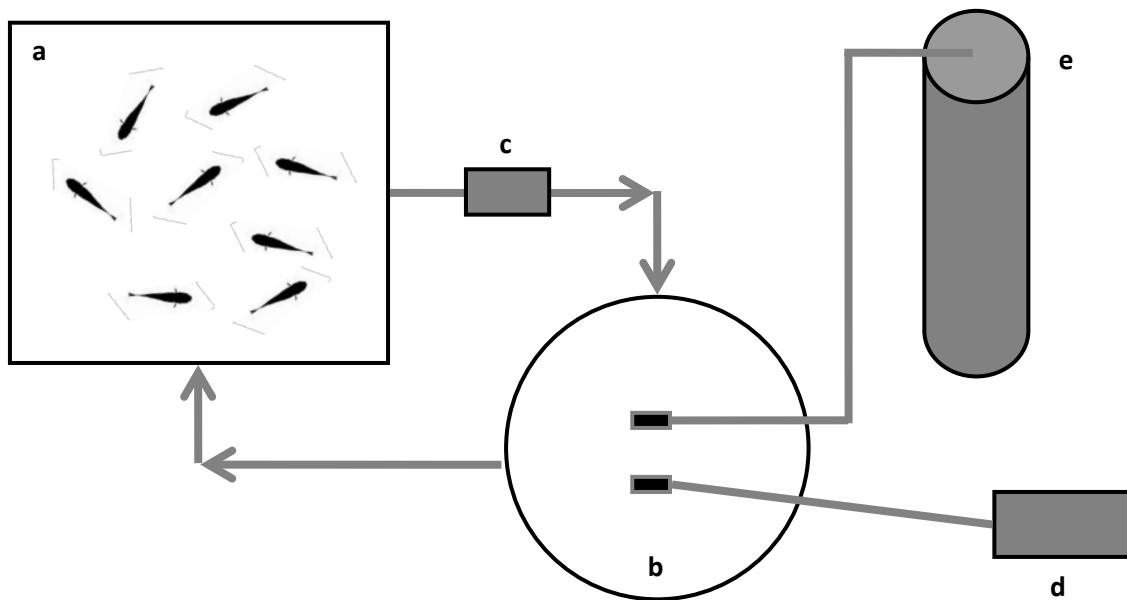
Fig. S1 – Scheme of the apparatus used for the experimental treatment. The subjects were inserted in the treatment tank (**a**; 45x50 cm, water depth 25 cm) in small groups; the experimental tank was connected to a cylindrical sump (**b**; 40 cm in diameter, 60 cm high, water level 50 cm) through a pump (**c**) that allowed a constant recirculation of the water; for the control treatment, an air pump (**d**) connected to an air stone in the sump ensure a stable oxygen saturation in the water (> 90 %); for the hypoxia treatment, a galvanic oxygen probe connected to an oxygen regulator and a solenoid valve allowed nitrogen flow from a nitrogen cylinder (**e**) to the air stones in the sump, allowing indirect regulation of oxygen saturation in the treatment tank.
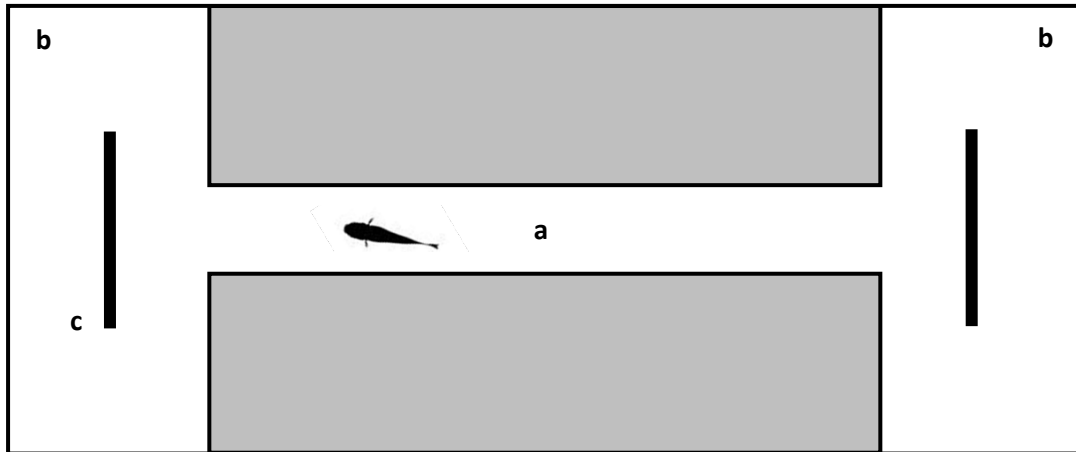
Fig. S2 – Scheme of the detour apparatus used for the lateralization assessment. The detour apparatus consisted in a 120x50 cm tank filled with 15 cm of water. A central runaway (75x10 cm) (**a**), made of white plastic material, connected two rectangular areas, one at each end of the tank (**b**). A plastic white and opaque barrier (**c**; 20 x 20 cm) was placed in front of each end of the runaway, at a distance of 6 cm. Immediately after completing the experimental treatment (*i.e.* hypoxia or normoxia), an individual subject was placed in one of the two rectangular areas of the apparatus (**b**), chosen at random; subsequently, the subject was encouraged to swim through the runway (**a**) using a pair of fish nets; the direction of the turn in front of the barrier (**c**) was recorded; each subject was observed during 10 left-right choice runs.