Zebrafish (*Danio rerio*) behavioral laterality predicts increased short-term avoidance memory but not stress-reactivity responses

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Abstract

Once considered a uniquely human attribute, behavioral laterality has proven to be ubiquitous among non-human animals, being frequently associated with different neurophenotypes in rodents and fish species. Zebrafish (*Danio rerio*) are a versatile and complementary vertebrate model system that has been widely used in translational neuropsychiatric research due their highly conserved genetic homology, well characterized physiological and extensive behavioral repertoire. Although the spontaneous left- and right-bias responses and associated behavioral domains (e.g. stress reactivity, aggression and learning) have previously been observed in other teleost species, no information regarding the natural left-right bias responses of zebrafish has been described. Thus, we aimed to investigate the existence and incidence of natural left-right bias of adult zebrafish in the Y-maze test and explore any relationship of biasedness on the performance of different behavioral domains. This included learning about threat-cues in the fear conditioning test and locomotion and anxiety-related behavior in the novel tank diving test. In conclusion, we showed for the first time that zebrafish exhibit a natural manifestation of behavioral lateralization which can influence aversive learning responses. Although laterality did not change locomotion or anxiety-related behaviors, we found that biased animals showed a reduction of short-term memory performance in the Y-maze and increase learning associated to fear cues.

Keywords: Anxiety; Behavioral asymmetries; Left-bias; Pavlovian fear conditioning; Right-bias.

1. Introduction

Lateralization of brain and behavior is the apparent predisposition towards side bias often manifested in terms of motor output, such as handedness, and has been widely studied in humans and animal models, including monkeys (Fagot and Vauclair 1991; Hopkins 1994; McGrew and Marchant 1997), rodents (Robison 1981; Rodriguez and Afonso 1993; Rodriguez et al. 1992), birds (Bhagavatula et al. 2014; Franklin and Adams 2010; Gunturkun et al. 1998) and fish (Bibost and Brown 2014; Bisazza and de Santi 2003; Dadda et al. 2010a; Dadda et al. 2010b). In rodents, several behavioral tasks have been used to assess behavioral asymmetries such turning rotometers, handedness, choice behavior, T-maze and Y-maze (Corballis 1986; Pisa and Szechtman 1986; Zimmerberg and Glick 1974). Variability in lateralization exerts a number of fitness benefits. For example, lateralization has been associated with maximization of brain processes, enabling individuals to process two tasks simultaneously (Rogers 2000; Rogers 2002). Moreover, studies have suggested that laterality evolved at the population level to maintain coordination among social groups (Rogers 2000).

Behavioral laterality is an evolutionarily conserved characteristic which is observed at populational level in humans and has been associated to different neurophenotypes (Corballis 2017; Frasnelli 2013). Behavioral asymmetries have been related to high escape performance (Dadda et al. 2010b), social responses (Reddon and Balshine 2010) and even accelerated learning responses (Andrade et al. 2001) in fish and rodent species. Behavioral laterality has also been implicated in anxiety and major depressive disorder (MDD) in humans, being cited as a factor in initiating and maintaining both disorders (Koster et al. 2010; Lichtenstein-Vidne et al. 2017). Interestingly, EEG studies showed that depressed and anxious patients exhibit abnormal alpha asymmetry, indicative of low right vs. left parietal activity, confirming that both disorders have a differential influence on lateralized hemispheric processing of emotional and verbal information (Bruder et al. 2016; Bruder et al. 1997; Henriques and Davidson 1990; Kentgen et al. 2000; Reid et al. 1998). Despite the clear relevance of lateralization to human neuropsychological function, we still have a limited understanding of the general origins of morphological and functional asymmetries in the brain and of their importance for behavior.

Zebrafish (*Danio rerio*) is a versatile vertebrate model system that has been widely used in translational neuropsychiatric research (Fontana et al. 2018; Stewart et al. 2015). The promise of zebrafish as an alternative organism for modeling human disorders is based on their conserved genome (Barbazuk et al. 2000; Howe et al. 2013; MacRae and Peterson 2015) and well-characterized physiology (Holzschuh et al. 2001; MacRae and Peterson 2015; Rico et al. 2011). In addition to its high genetic and physiological homology with mammals, zebrafish presents a well-conserved behavioral repertoire (Kalueff et al. 2013) which may be useful in increasing our understanding of the evolutionary origins and functional relevance of left-right asymmetry. Thus, zebrafish have much potential for characterizing the mechanisms involved in behavioral laterality. Although the spontaneous left- and right-bias responses and its correlation to other behavioral domains (e.g. stress reactivity, aggression and learning) were previously observed in other teleost fish species (Ariyomo et al. 2013; Bibost and Brown 2014; Byrnes et al. 2016), no information regarding zebrafishes natural left-right bias responses was previously described. Thus, here we aimed to investigate the existence and incidence of natural left-right bias of adult zebrafish in the Y-maze test and explore how this tendency relates to performance on different behavioral domains, including learning about threat-cues in the fear conditioning test and, locomotion and anxiety-related behavior in the novel diving tank.

2. Material and Methods

2.1. Animals

Adult zebrafish (~ 50:50 male: female ratio at 3-month of age) were bred in-house and reared in standard laboratory conditions on a re-circulating system (Aquaneering, USA). Animals were maintained on a 14/10-hour light/dark cycle (lights on at 9:00 a.m.), pH 8.4, at ~28.5 °C (±1 °C) in groups of 20 animals per 2.8 L. Fish were fed three times/day with a mixture of live brine shrimp and flake food, except in the weekend that they were fed once/day. Animals were tested in the Y-maze apparatus and pair-housed for 24 hours for further analysis of shock-avoidance response to reduce stress from multiple handling in a single day (see **Fig. 1**). After behavioral tests, all animals were euthanized using 2-phenoxyethanol from Aqua-Sed (Aqua-Sed™, Vetark,Winchester, UK).

2.2. Y-maze test

The Y-maze spontaneous alternation task is widely used for measuring the disposition of different animal models to explore new environments and to assess left- and right-biased responses (Barnard et al. 2016; Castellano et al. 1987; Frasnelli 2013; Rodriguez et al. 1992). One-hundred and one adult zebrafish were used for assessing Y-maze performance and right-left bias. Required sample size was calculated *a priori* following pilot tests (effect size (d) = 0.3, power = 0.8, alpha = 0.05). The apparatus consisted of a white Y-maze tank with three identical arms (5 cm length x 2 cm width; three identical arms at a 120° angle from each other) and a transparent base, filled with 3L of aquarium water (**Fig. 1**). Ambient light allowed some visibility in the maze, but no explicit intra-maze cues were added to the environment. Behavioral tests were performed between 10 a.m. to 4 p.m. using the Zantiks (Zantiks Ltd., Cambridge, UK) AD system (Brock et al. 2017), and we carried out three independent replicates. The Zantiks AD system was fully controlled via a web-enabled device during behavioral training. Fish behavior was recorded for 1 hour and was assessed according to overlapping series of four choices (tetragrams) and analyzed as a proportion of the total number of turns (Gross et al. 2011). The relative number of repetitions (rrrr + llll), alternations (rlrl + lrlr), right turns and left turns were also calculated as a proportion of the total number of turns. The mean and coefficient of variation for right and left bias was calculated for each animal and behavioral lateralization was considered when the animal presented >60% of preference for right or left Y-maze arm.

2.3. Pavlovian fear conditioning

The inhibitory avoidance paradigm is a valid method widely used to explore mechanisms underlying fear avoidance learning responses in zebrafish (Amorim et al. 2017; Manuel et al. 2014; Manuel et al. 2015; Ng et al. 2012). 24h after the completion of the Y-maze test, animals (*n*= 55) were tested on a Pavlovian fear conditioning procedure for 1 hour. The fear conditioning response was based on previous work (Cleal and Parker 2018; Valente et al. 2012). Fish were individually placed in one of four lanes of a tank (25 cm length x 15 cm, 1 L of water in each tank) (**Fig. 1**). Briefly, fish were habituated for 30 minutes into the test environment, half check and half grey base switching position every 5-min. The baseline preference was ascertained over 10-min and only the last 10-min baseline preference was used for assessing the area preference. Conditioning phase was followed, which consisted in a conditioned stimulus (CS+; full screen of “check” or “grey”, randomized between each batch) presented for 1.5s and followed by a brief mild shock (9 V DC, 80ms; unconditioned stimulus (US). After this, an 8.5s of inter-trial interval (ITI) of the non-CS (CS−) exemplar was presented at the bottom of the tank. The CS+/US was exhibit nine times. Finally, avoidance of CS+ was assessed by presenting the baseline screen (CS+ and CS− simultaneously) for 1-min, and switching positions after 30-s.

2.4. Novel tank diving test

The novel tank diving test is commonly used for analyzing locomotor and anxiety-like phenotypes in zebrafish presenting a high sensitivity to anxiolytic and anxiogenic drugs (Egan et al. 2009; Kalueff et al. 2013; Levin et al. 2007; Maximino et al. 2010; Mezzomo et al. 2016; Wong et al. 2010). 24 hours after the Y-maze test, animals (*n = 46*) were placed individually in a novel tank (30 cm length x 15 cm height x 12 cm width) containing 4 L of aquarium water. Behavioral activity was recorded using 2 webcams (front and top view, see **Fig. 1B**) for 5 min to analyze thigmotaxis and diving response (Egan et al. 2009; Parker et al. 2012; Rosemberg et al. 2012). Behaviors were measured in an automated video-tracking software (EthoVision, Noldus Information Technology Inc., Leesburg, VA - USA) at a rate of 60 frames/s. The tank was separated in three virtual areas (bottom, middle and top) to provide a detailed evaluation of vertical activity. The following endpoints were measured: total distance traveled, time spent in each third of the tank, immobility and thigmotaxis.

2.5. Randomization and blinding

All behavioral testing was carried out in a fully randomized order, choosing fish at random from one of ten housing tanks for testing. Fish were screened for left-right bias in the Y-maze first, but analysis was not carried out prior to subsequent behavioral testing to avoid bias. Subsequent to Y-maze screening, fish were pair housed and issued a subject ID, allowing all testing to be carried out in a fully blinded manner. Once all data were collected and screened for extreme outliers (e.g., fish freezing and returning values of ‘0’ for behavioral parameters indicating non-engagement), the bias was revealed and data analyzed in full.

2.6. Data reduction and statistical analysis

Data obtained from the Y-maze protocol was reduced using the Zantiks Y-maze Analysis Script created especially for this purpose (available from: https://github.com/thejamesclay/ZANTIKS_YMaze_Analysis_Script). Subsequently, data were analyzed in IBM SPSS® Statistics and the results were expressed as means ± standard error of the mean (S.E.M). To assess whether there were any effects of bias on total turns, alternations (lrlr + rlrl), repetitions (rrrr + llll) and novel tank responses we used linear mixed effects model (Poisson distribution, log link), with bias and time as fixed factors, and ID as a random factor (to account for non-independence of replicates). Additionally, left-right bias effects on shock avoidance test was assessed using two-way repeated measures ANOVA with ‘bias’ (left vs right vs neutral) and conditioning (pre-vs-post) as factors, and preference for conditioned stimulus as the dependent variable. Newman-Keuls test was used as post-hoc analysis, and results were considered significant when $p \leq 0.05$.

3. Results

2.1. Left-right bias profile in the Y-maze test

Zebrafish showed behavioral lateralization in the Y-maze (right-biased 27.18 %, left-biased 27.18% and non-biased 45.63%). To confirm if the behavioral laterality was consistent across time, the coefficient of variation for the left and right turn preferences were calculated for the non-biased (19.28 ± 2.52 and 21.05 ± 2.95), left-biased (30.40 ± 3.85 and 21.7 ± 3.61) and right-biased (27.23 ± 5.52 and 25.95 ± 2.31) groups. **Figure 2** displays the Y-maze data. A significant bias effect was observed for number of turns ($F_{(2, 601)} = 13.115$; $p<0.0001$), repetitions ($F_{(2, 601)} = 39.696$; $p<0.0001$) and alternations ($F_{(2, 601)} = 45.437$; $p<0.0001$). A time effect (data not shown) was also observed for number of turns ($F_{(5, 601)} = 9.769$; $p<0.0001$), repetitions ($F_{(5, 601)} = 3.242$; $p=0.007$) and alternations ($F_{(5, 601)} = 3.801$; $p=0.002$). Additionally, a significant interaction effect (bias * time) was observed for repetitions ($F_{(10, 601)} = 2.504$; $p=0.006$) and alternations ($F_{(10, 601)} = 2.390$; $p= 0.009$). Right-biased significantly increased the number of repetitions ($p<0.0001$) and, decreased the number of turns ($p<0.05$) and alternations ($p<0.0001$) compared to non-biased animals. Meanwhile, left-biased animals had an increased number of repetitions ($p<0.001$) and decreased alternations ($p<0.005$) compared to non-biased group. Moreover, right-biased animals had a significant increase of repetitions ($p<0.05$) and decrease of alternations ($p<0.05$) even when compared to left-biased animals (**Fig. 2A**). The behavioral profile of biased and non-biased animals is displayed in **Fig. 2B** tetragrams where a high number of llll and rrrr configuration can be observed for left- and right-biased animals, respectively.

2.2. Short-term avoidance memory and novelty response of biased animals

Although no interaction effect bias *vs.* shock ($F_{(2, 98)} =1.259$; $p= 0.312$) was observed for the Pavlovian responses, a significant effect for bias ($F_{(2, 98)} =3.128$; $p= 0.035$) and shock ($F_{(1, 98)} = 79.47$; $p<0001$) effect was founded. ANOVA often is underpowered to detect the significance of interaction terms (Wahlsten 1990), and therefore further analysis was performed to specifically analyze interaction effects. Concerning this, all biased and non-biased animals had a decreased time spent in the preference for conditioned stimulus. Moreover, both left- and right- biased animals ($p<0.05$) had a significantly decreased time spent in the conditioned area compared to non-biased group (**Fig. 3**). No significant effect was observed for bias in all novel tank diving test-related parameters, including distance travelled ($F_{(2, 43)} = 0.683$; $p = 0.510$), immobility ($F_{(2, 43)} = 2.348$; $p = 0.107$), time in tank zones time ($F_{(2, 129)} = 0.084$; $p = 0.918$) (**Fig. 4**) and thigmotaxis ($F_{(2, 43)} = 1.289$; $p = 0.286$) (**Fig. 5**).

4. Discussion

In this study we evaluated left-right bias from unconditioned Y-maze performance and evaluated the predictive validity of Y-maze performance on both unconditioned and conditioned measures of fear/anxiety. We showed, for the first time, that the zebrafish presents natural behavioral laterality in the Y-maze test, suggesting that the protocol may be useful for screening this species for behavioral asymmetry. Second, we found that behaviorally lateralized animals show decreased pure alternation responses in the Y-maze, and increased pure repetition, suggesting that learning and memory may be partially inhibited in lateralized animals. Third, we observed that behavioral asymmetry predicts increased learning in a Pavlovian fear conditioning protocol but did not predict measures of unconditioned anxiety (novel tank test, thigmotaxis). Collectively, these data suggest, contrary to theories that laterality is related to increased stress-reactivity, that increased behavioral laterality may be related to increased cue-reactivity, particularly in relation to aversive cues. This has connotations for translational models of human disorders of affective state, in which heightened attention to threat-related cues is observed (Lichtenstein-Vidne et al. 2017) and in which brain and behavioral laterality are thought to be risk factors (Bruder et al. 2016).

Left-right asymmetries in behavioral protocols including the T-maze and Y-maze have been widely utilized in rodents (Andrade et al. 2001; Nakagawa et al. 2004; Rodriguez et al. 1992). Here, for the first time, we observed that approximately a quarter of zebrafish present substantial natural left- (27.18 %) or right- (27.18%) locomotor lateralization, with the remaining 45.63% animals showing stochastic patterns of left/right. These data are somewhat at odds from observed bias in rodent models, in which there is a high number of right-biased animals (52.8%) and low numbers of of left- (22.2%) and non-biased responses (25%) (Andrade et al., 2001).

We observed that behavioral laterality has an important role in Y-maze performance, where left-right biased animals presented an increase of repetition behavior and decrease of alternation, in particular in the left-biased animals. The relative frequency of pure alternations (lrlr, rlrl) to pure repetitions (llll, rrrr) is thought to be a measure of short-term memory (Cleal and Parker 2018; Gross et al. 2011). Alterations have been directly associated with functionally distinct search patterns, where the seeking for change and novelty may have a role in their exploratory profiles (Kool et al. 2010). Interestingly, decreases in pure alternations and increases in pure repetition behavior in lateralized animals was first described by Rodriguez et al. (1992), who demonstrated that both stress and over-training decrease the alternation/repetition ratio through the promotion of an increase of biased responses. Overall, we confirmed that both alternations and repetitions remain as a highly reliable behavioral pattern that is conserved across species (Ghafouri et al. 2016; Lewis et al. 2017; Pickering et al. 2015).

In agreement with previously studies (Andrade et al. 2001), we showed that locomotor lateralization is associated to increased learning in a Pavolvian fear conditioning protocol. This appears, initially, to be at odds with our observations regarding memory in the Y-maze (operationally defined as variation in alternation/repetition). However, the most predominant theory of how left-right bias affects learning and cognitive processing relates to a hypothesized increased stress-reactivity in lateralized animals (Carlson and Glick 1989; Neveu 1996; Westergaard et al. 2001). Interindividual differences in laterality have been shown to covary with, or predict, individual differences in stress-reactivity and susceptibility to stress-related pathology (Byrnes et al. 2016; Carlson and Glick 1989; Fride and Weinstock 1989; Ocklenburg et al. 2016). Here, we tested the hypothesis that left- and right-biased animals would differ in measures of stress-reactivity and anxiety-like phenotypes (Blaser and Rosemberg 2012; Egan et al. 2009; Parker et al. 2012). We found no significant differences in lateralized animals in our measures of anxiety, suggesting that the observed differences in behavioral phenotypes observed in the Y-maze and shock avoidance learning seems to not be related to stress-reactivity responses *per se*. Instead, our data may suggest that the lateralized fish are more reactive to stress-related cues. This would explain the increased performance on the Pavlovian fear conditioning, the fact that there were no differences in measures of anxiety, and the reduced memory on the Y-maze test.

There are several theories regarding the mechanisms underlining behavior laterality in simple maze tasks. Diaz Palarea et al. (1987) were the first to report that left-right biased animals, as assessed via spatial asymmetry in a T-maze, had alterations in dopaminergic (DA) signaling. In addition, apomorphine (DA receptor agonist) and 6-hydroxydopamine lesions alters behavioral laterality of animals in the T-maze test (Castellano et al. 1987) and Y-maze (Nakagawa et al. 2004), confirming the involvement of DA system in behavioral asymmetry. DA receptors are strongly implicated in emotional learning and recall of emotionally relevant events in rats. For example, activation of D4-receptors in the medial pre-frontal cortex potentiates fear-associated memory formation but has no impact on recall (Lauzon et al. 2009; Laviolette et al. 2005), whereas activation of D1-like receptors blocks recalls of previously learned fear-associated memories but has no impact on learning (Lauzon et al. 2009), suggesting a double dissociation of function. Interestingly, the serotonergic (5-HT) system has also been shown to have an important role in mediating individual differences in anxiety-like responses and locomotor activity in zebrafish and exerts a minor modulatory role of the DA system (Tran et al. 2016). Both behavioral laterality and aversive memory is mostly associated with modulatory action of DA system, but the 5-HT system has a major role modulating zebrafish responses to novelty. The precise mechanisms of how behavioral laterality modulates neuropsychiatric conditions are yet to be firmly established, and further studies are required to better understand the mechanisms in which behavioral laterality modulates aversive memory in zebrafish.

5. Conclusion

Overall, we showed for the first time that zebrafish exhibits natural manifestation of behavioral lateralization and it can influence on aversive learning responses. We found that bias animals show a reduction of short-term memory performance in the Y-maze, but an increase in learning in a Pavlovian fear conditioning protocol. Coupled with a lack of differences between lateralized and non-lateralized animals in unconditioned tests of anxiety, our data suggest that lateralized zebrafish may show heighted reactivity to fear related cues. These results have important connotations for translational models of depression and anxiety, particularly in the light of well-established links between laterality and anxiety/depression in humans. Finally, because biased animals present different behavioral performances in the Y-maze and Pavlovian fear conditioning protocols, left- and right- preference should be considered when working with zebrafish behavior, particularly to control variability in performance on more complex tasks.

Compliance with Ethical Standards

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Conflict of interest

The authors declare that no conflict of interest exists.

Ethical approval

All experiments were carried out following scrutiny by the University of Portsmouth Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F].

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Figure Captions

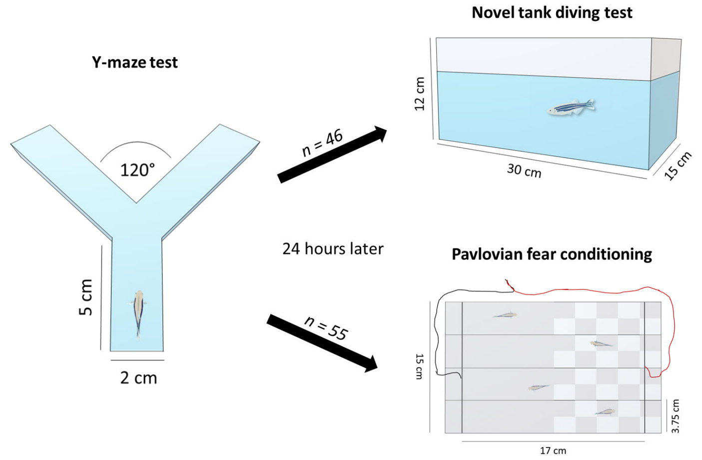
Figure 1. Schematic representation of the experimental design and the behavioral tasks.

Figure 2. Effects of left- and right- bias in zebrafish on the Y-maze test. (A) Laterality affects total number of turns, repetitions and alternation of adult zebrafish. (B) Y-maze tetragrams showing the behavioral phenotype of biased and non-biased animals. Data were represented as mean ± S.E.M. and analyzed by linear mixed effects, followed by Tukey's multiple comparison test. Asterisks indicates statistical differences compared to non-biased group or between biased groups (*p < 0.05, **p <0.01, ***p <0.001 and *p<0.0001, n = 47 non-biased, n = 28 left-biased and n = 28 right-biased group).

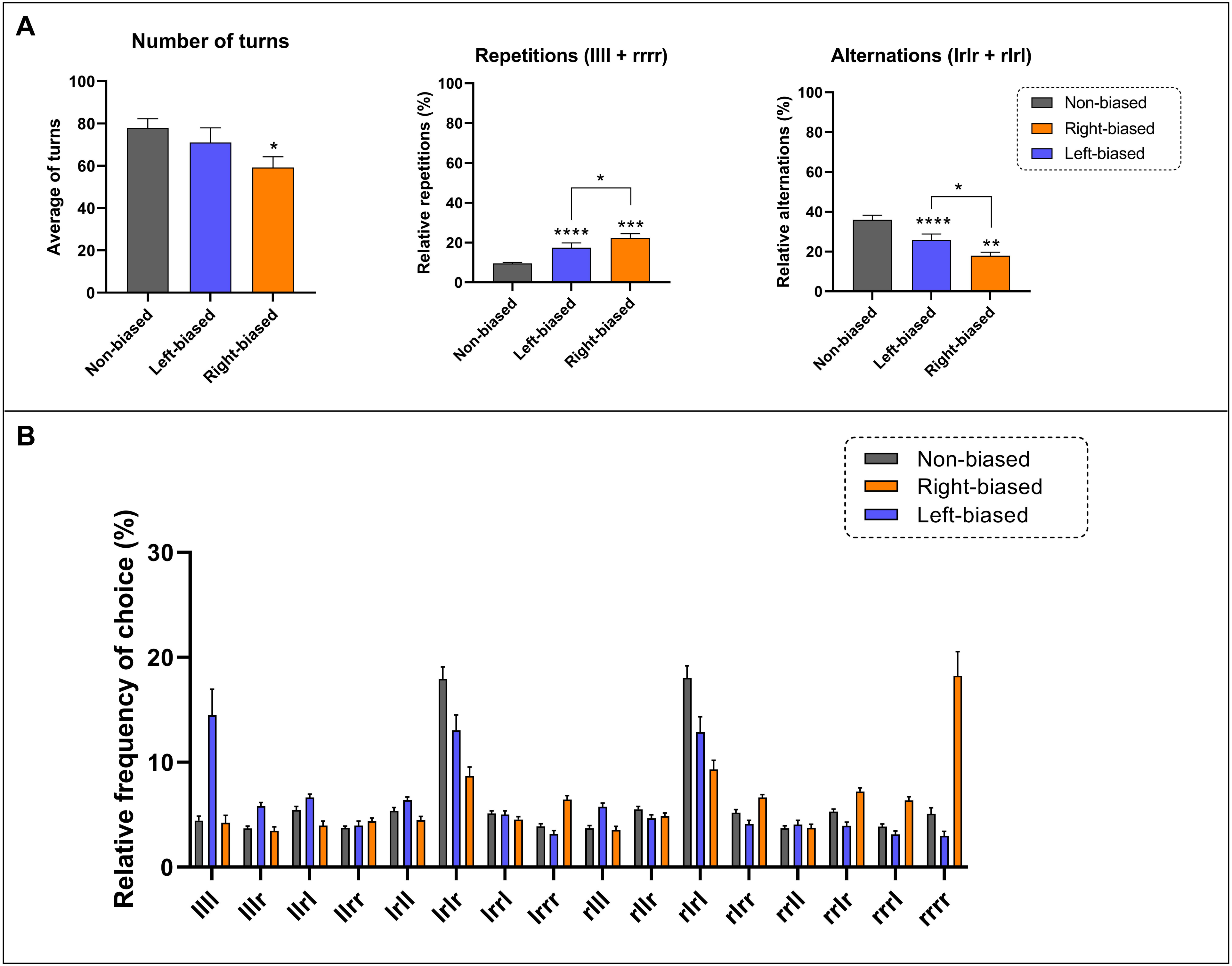
Figure 3. Left- and right- bias are related to fear avoidance learning responses in adult zebrafish. Data were represented as mean ± S.E.M. and analyzed by two-way RM ANOVA, followed by Tukey's multiple comparison test. Asterisks indicates statistical differences compared to non-biased group or between biased groups (**p* < 0.05, ***p* <0.01, ****p* <0.001 and **p*<0.0001, *n* = 25 non-biased, *n* = 17 left-biased and *n* = 13 right-biased group).

Figure 4. Behavioral laterality is not related to locomotor or anxiety-related phenotypes in adult zebrafish. Data were represented as mean ± S.E.M. and analyzed by linear mixed effects (*n* = 22 non-biased, *n* = 11 left-biased and *n* = 15 right-biased group).

Figure 5. Left- and right- bias do not change thigmotaxis in adult zebrafish. Data were represented as mean ± S.E.M. and analyzed by linear mixed effects (*n* = 20 non-biased, *n* = 11 left-biased and *n* = 15 right-biased group).



Y-maze test
120°
5 cm
2 cm
n = 46
n = 55
24 hours later
Novel tank diving test
12 cm
30 cm
15 cm
Pavlovian fear conditioning
15 cm
3.75 cm
17 cm


A
Number of turns
Average of turns
100
80
60
40
20
0
*
Non-biased
Left-biased
Right-biased
Repetitions (llll + rrrr)
Relative repetitions (%)
100
80
60
40
20
0
*
****
***
Non-biased
Left-biased
Right-biased
Alternations (lrlr + rlrl)
Relative alternations (%)
100
80
60
40
20
0
*
****
**
Non-biased
Left-biased
Right-biased
Non-biased
Right-biased
Left-biased
B
Non-biased
Right-biased
Left-biased
Relative frequency of choice (%)
30
20
10
0
llll
lllr
llrl
llrr
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lrrl
lrrr
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rlrr
rrll
rrlr
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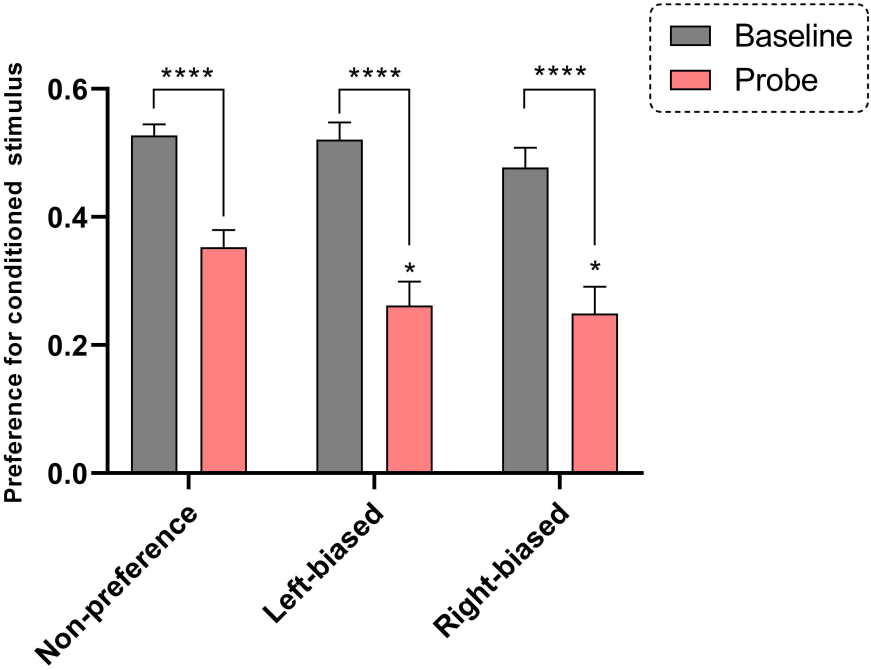
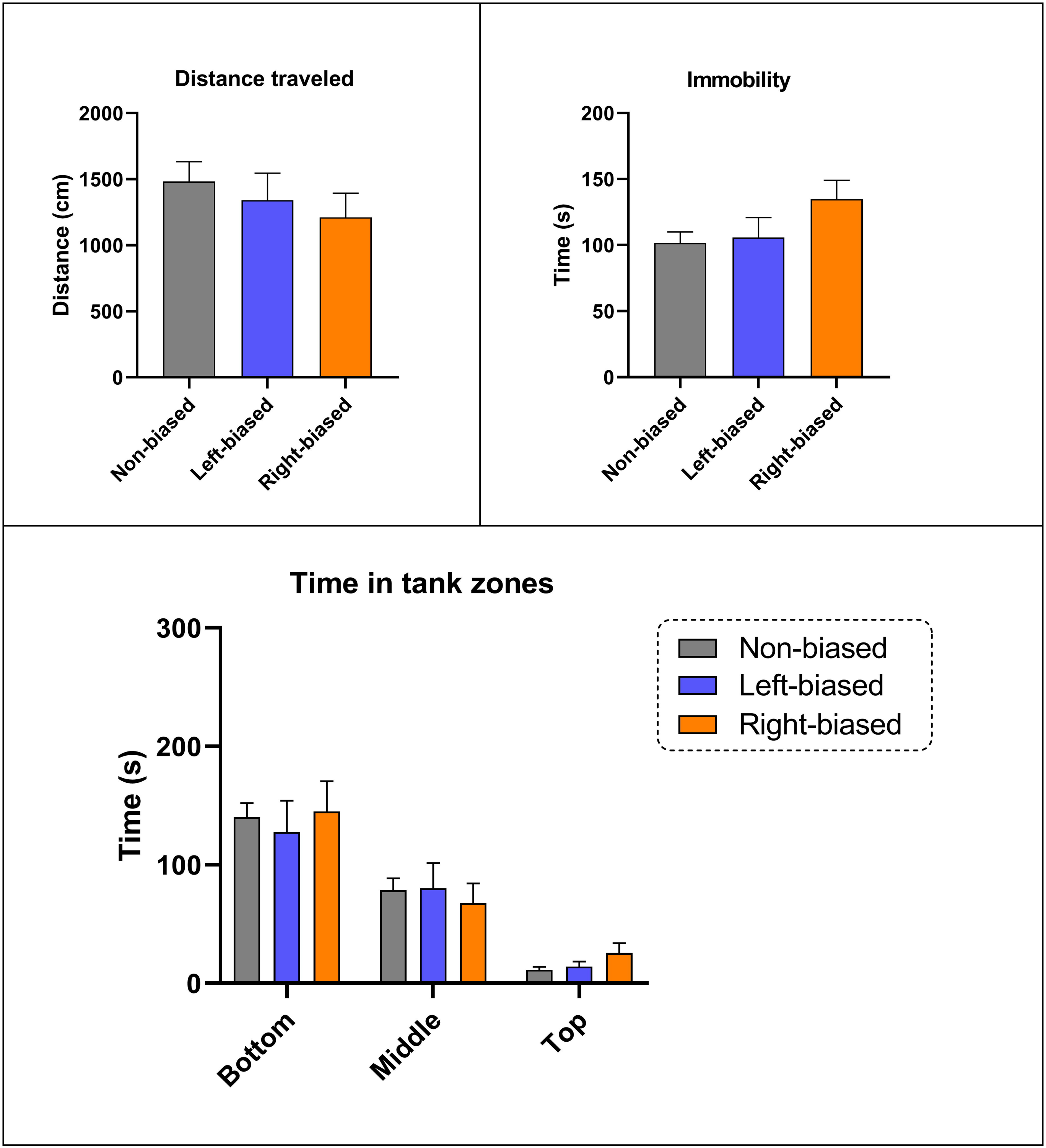
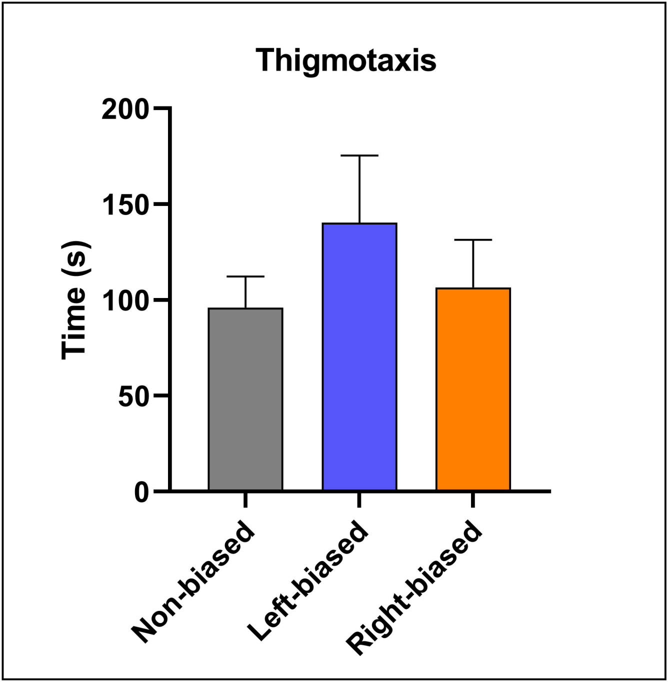

Baseline
Probe
Preference for conditioned stimulus
0.0
0.2
0.4
0.6
****
****
****
*
*
Non-preference
Left-biased
Right-biased


Distance traveled
Distance (cm)
2000
1500
1000
500
0
Non-biased
Left-biased
Right-biased
Immobility
Time (s)
200
150
100
50
0
Non-biased
Left-biased
Right-biased
Time in tank zones
Time (s)
300
200
100
0
Bottom
Middle
Top
Non-biased
Left-biased
Right-biased


Thigmotaxis
Time (s)
200
150
100
50
0
Non-biased
Left-biased
Right-biased