

**Female adult zebrafish (*Danio rerio*) show higher levels of anxiety-like behavior than males, but do not differ in learning and memory capacity**

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## Abstract

Zebrafish (*Danio rerio*) are widely used as a translational model for human neuropsychiatric conditions. Many studies have not considered sex differences in their analyses. Here, we studied sex differences of adult zebrafish in two behavioral domains: Anxiety and Memory. To assess whether sex influences anxiety-like responses, we used two different behavioral protocols, the novel tank diving task and the light-dark test. To assess sex differences in learning and memory tasks, we explored two memory domains, short-term spatial memory (free movement pattern Y-maze task) and short-term fear memory (Pavlovian fear conditioning task). Although we did not find any significant difference in learning and memory tasks, female zebrafish showed robust increases in anxiety-like behavioral endpoints in both anxiety tests. Overall, our data suggest that zebrafish is a sensitive model to work with sex differences when modeling anxiety-related disorders and this should be an important factor to consider in different experimental designs.

**Keywords:** Light-dark test; Novel tank diving test; Pavlovian fear conditioning test; Sex differences; free movement pattern Y-maze task.

## 1. Introduction

Sex differences affect development and behavior of several species (Rose & Rudolph, 2006; Mowrey & Portman, 2012), extending to psychiatric disorders which have variations in their associated phenotypes and levels of expression for females vs males (Bekker & van Mens-Verhulst, 2007; Laurin *et al.*, 2007; Mandy *et al.*, 2012). Zebrafish is widely used as a translational model to understand neuropsychiatric disorders including neurodevelopmental (e.g. autism and schizophrenia)(Morris, 2009; Kim *et al.*, 2014; Kozol *et al.*, 2015; Chen *et al.*, 2018) and anxiety-related disorders (Piato *et al.*, 2011; Stewart *et al.*, 2012; Chakravarty *et al.*, 2013; Ziv *et al.*, 2013). Thus, in order to ensure their translational relevance to humans, studies have been developed to better understand the behavioral sex differences in zebrafish (Lopez Patino *et al.*, 2008; McCurley & Callard, 2008; Dahlbom *et al.*, 2012; Ariyomo *et al.*, 2013; Tran & Gerlai, 2013). For example, Rambo *et al.* (2017) showed that females are more aggressive than males in the mirror-induced aggression test. There are more subtle sex differences in social behavior in zebrafish, where males prefer to approach one single female instead of a group of males in the social preference test (Ruhl *et al.*, 2009). To date, however, there have been no specific studies into sex differences in zebrafish in two behavioral domains that are very important in translational neuroscience: anxiety and learning/memory.

Anxiety-related phenotypes have been measured in several different protocols in zebrafish, including the novel tank diving task and the light-dark task (Levin *et al.*, 2007; Egan *et al.*, 2009; Maximino *et al.*, 2010a; Maximino *et al.*, 2010b; Wong *et al.*, 2010; Rosemberg *et al.*, 2011; Blaser & Rosemberg, 2012; Parker *et al.*, 2012; Kalueff *et al.*, 2013; Mezzomo *et al.*, 2016). The novel tank diving test is commonly used for analyzing locomotion and vertical activity, both of which are linked to zebrafish anxiety-like behavior owing to high sensitivity to anxiolytic and anxiogenic drugs (Levin *et al.*, 2007; Egan *et al.*, 2009; Wong *et al.*, 2010; Parker *et al.*, 2012). The light-dark test is a behavioral task widely used to assess fear- and anxiety-like responses, which explore the natural tendency of zebrafish to avoid brightly lit environments (Maximino *et al.*, 2010a; Rosemberg *et al.*, 2011; Blaser & Rosemberg, 2012). None of the above studies have specifically examined sex differences, nor are sex differences reported in the papers. In rodents, sex differences in anxiety have been identified. For example, males are less active and defecate more in the open field (Blizard *et al.*, 1975). In addition, many papers using the elevated-plus maze have demonstrated that females spend more time in in the open arms, suggesting lower anxiety-like behavior (Johnston & File, 1991; Xiang *et al.*, 2011).

Regarding memory and cognition, different types of memory are related to different patterns of behavior in neuropsychiatric disorders including spatial memory (Mizoguchi *et al.*, 2000; Myles-Worsley & Park, 2002) and conditioned fear (Bortz, 2003; Dere *et al.*, 2010). Several protocols have been used to assess memory in zebrafish, including the free movement pattern (FMP) Y-maze task (Cleal & Parker, 2018) and Pavlovian fear conditioning (Valente *et al.*, 2012; Brock *et al.*, 2017), but none included analysis of sex differences. The FMP Y-maze task is widely used for measuring short-term spatial memory and behavioral laterality (Gross *et al.*, 2011; McDowell *et al.*, 2016; Cleal & Parker, 2018). The inhibitory avoidance paradigm is used to explore mechanisms underlying fear avoidance learning in zebrafish (Ng *et al.*, 2012; Manuel *et al.*, 2014; Manuel *et al.*, 2015; Amorim *et al.*, 2017). Although female rats perform better than males in several learning and memory-related tasks such as the classical eyeblink conditioning, in fear-potentiated startle and in most operant conditioning tasks (Dalla & Shors, 2009), in none of the zebrafish papers mentioned above have sex differences been evaluated or reported.

There is a critical need to establish if there are sex differences in behavioral performance in zebrafish in anxiety and learning/memory tests. In the present study, we sought to examine potential sex differences in both behavioral domains, anxiety (novel tank and light-dark test) and learning/memory (Pavlovian fear conditioning task and FMP Y-maze task), using a suite of established behavioral protocols.

## 2. Materials and Methods

### 2.1. Animals

Adult zebrafish (AB wild-type; ~ 30:33 male: female ratio at 4-month of age) were bred in-house and reared in standard laboratory conditions on a re-circulating system (Aquaneering). Animals were maintained on a 14/10-hour light/dark cycle (lights on at 9:00 a.m.) at ~28.5 °C ( $\pm 1$  °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 weekends where they were fed once/day. Required sample sizes of ~30 for each protocol ( $n = 15$  M +  $n = 15$  F) were calculated *a priori* following pilot and previous observations in our laboratory of sex differences in novel tank test ( $d = 1.25$ , power = 0.8, alpha = 0.05). For learning and memory, we observed no significant differences between the sexes in our initial experiments (F values ~1), and owing to the novelty of this task for sex

differences, we increased the sample size to include a further 6 fish ( $n = 3$  male;  $n = 3$  female) to be conservative.

Animals were tested in the light-dark or novel tank test, and then pair-housed for 24 hours for further analysis of shock-avoidance or FMP Y-maze task (see **Fig. 1**). The pair-housing system was chosen based on previous studies (Parker *et al.*, 2012), in which the pair-housing system reduces the stress induced by social isolation of zebrafish while still allowing for individual identification. During the pair-housing period, animals had shared water system and visual contact through transparent partition. Importantly, males were pair-housed only with females once that male-male interactions to avoid aggressive behavior and establishment of dominance hierarchies (Spencer & Smith, 2005; Paull *et al.*, 2010; Dahlbom *et al.*, 2012; Teles & Oliveira, 2016). Behavioral tests were performed between 10 a.m. to 4 p.m in three experimental replicates. All testing was fully automated to further increase reliability. We used multiple protocols for each domain to ensure intra-trait reliability. After behavioral tests, animals were euthanized using 2-phenoxyethanol from Aqua-Sed (Aqua-Sed™, Vetark, Winchester, UK). Following data collection, sex differentiation was made by four trained researchers (inter-rater reliability 0.76 with percent overall agreement = 82.35%) based on fish body shape, color patterns and presence of prominent genital papilla according to Yossa *et al.*, (2013). 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].

## 2.2. Randomization and blinding

All behavioral tests were carried out in a fully randomized order, choosing fish at random from one of twelve groups for testing. Fish were screened for anxiety-like behavior (light-dark or novel tank diving tasks) first, then subsequently pair-housed and issued a subject ID. 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 zebrafish sex was revealed, and data analyzed in full.

## 2.3. Novel tank diving test

Animals ( $n = 30$ ) were placed individually in a novel tank (20 cm length x 17.5 cm height x 5 cm width) containing 1 L of aquarium water. Behavioral activity was analyzed using the

Zantiks automatic system (Zantiks Ltd., Cambridge, UK) for 5 minutes (Egan *et al.*, 2009; Parker *et al.*, 2012; Rosemberg *et al.*, 2012). The Zantiks AD system was fully controlled via a web-enabled device during behavioral training. 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, and time spent in each third of the tank.

#### 2.4. Light-dark test

The light-dark test was performed in a black tank (20 cm length x 15 cm height x 15 cm width) divided into two equally sized partitions where half of the tank area contained a bright white light and the other area was covered with a black partition to avoid light exposure (see **Fig 1**). Animals ( $n= 30$ ) were placed individually into the behavioral apparatus and its activity was analyzed using the Zantiks automatic system (Zantiks Ltd., Cambridge, UK) for 5 minutes to determine the time spent in dark area (Maximino *et al.*, 2010a; Blaser & Rosemberg, 2012; Mezzomo *et al.*, 2016).

#### 2.5. FMP Y-maze task

Adult zebrafish ( $n= 36$ ) were used for assessing sex differences in the FMP Y-maze task. The apparatus consisted in a white Y-maze tank with three identical arms (5 cm length x 2 cm width) and a transparent base, filled with 3L of aquarium water (**Fig. 1**). No explicit intra-maze cues were added to the environment and ambient light allowed some visibility in the maze. Fish behavioral patterns in the Y-maze were assessed using the Zantiks (Zantiks Ltd., Cambridge, UK) AD system (Brock *et al.*, 2017). 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). To assess short-term spatial memory, the relative number of repetitions (rrrr + llll) and alternations (rlrl + lrlr) were also calculated as a proportion of the total number of turns. Further analysis to see behavioral laterality was conducted considering the number of left- and right-turns across 1 hour, the behavioral laterality was considered when animals had  $60 \geq$  of left- or right-biased responses.

#### 2.8. Pavlovian fear conditioning

Zebrafish ( $n= 36$ ) were tested on a Pavlovian fear conditioning procedure for 1 hour based on previous work (Valente *et al.*, 2012; Brock *et al.*, 2017; Cleal & Parker, 2018). Fish were

individually placed in one of four lanes of a tank (25 cm length x 15 cm, 1 L of water) (**Fig. 1**). Briefly, fish were habituated for 30 minutes into the test environment, which comprised a half check and half grey base screen, the position of which switched every 5-min. Baseline preference was established over 30-min and only the last 10-min baseline preference was used for assessing the time spent in the tank areas. Baseline was followed by a conditioning phase in which a conditioned stimulus (CS+; full screen of “check” or “grey”, randomized between each batch) was presented for 1.5-s and followed by a brief mild shock (9 V DC, 80ms; unconditioned stimulus (US). After this, an 8.5-s of inter-trial interval (ITI) of the non-CS (CS-) exemplar was presented at the bottom of the tank. The CS+/US was repeated nine times. Lastly, avoidance of CS+ was assessed by presenting the baseline screen (CS+ and CS- simultaneously) for 1-min, and switching positions after 30-s. The retention index was calculated by the following formula:

$$\text{retention index} = (\text{baseline} - \text{probe}) - 1$$

## 2.9. Statistics

Data were analyzed in IBM SPSS® Statistics and the results were expressed as means  $\pm$  standard error of the mean (S.E.M). To assess whether there were any effects of sex on total turns, alternations (lrlr + rlrl) and repetitions (rrrr + llll) we used linear mixed effects model (Poisson distribution, log link), with sex and time as fixed factors, and ID as a random factor (to account for non-independence of replicates). Novel tank vertical activity was also assessed using linear mixed effects with ‘sex’ (male vs female) and zones (bottom, middle and top) as factors. Finally, to assess whether there were any effects of sex on distance traveled, time spent in the dark and retention index, we used a Student’s T-Tests. Tukey’s test was used as post-hoc analysis, and results were considered significant when  $p \leq 0.05$ . The inter-rater reliability was measured by using the multiple raters Kappa (Fleiss Kappa) test.

## 3. Results

### 3.1. Females shows higher levels of anxiety-like behavior in two different behavioral tasks

**Figure 2** shows the sex differences in the novel tank diving task and light-dark test. In the novel tank test, a significant sex\*zone interaction effect was observed for time spent in tank zones ( $F_{(2, 87)} = 17.00$ ;  $p=0.0001$ ) without any significant change for the distance traveled ([male vs female]  $t_{(28)} = 0.005$ ;  $p = 0.99$ ). Post-hoc analysis showed that females spend more time in the bottom zone and less time in the top zone compared to males (**Fig. 2A**). In the light-dark test, a

significant sex effect for the time spent in the dark zone was observed ([male vs female]  $t_{(26)} = 2.329$ ;  $p = 0.0279$ ), where females spent more time in the dark zone compared to males.

### 3.2. Sex does not have a role in zebrafish Pavlovian responses and short-term spatial memory

**Figure 3** displays the patterns of behavior in two cognition and memory-related tests. In the FMP Y-maze task, no significant sex effect across time was observed for total turns ( $F_{(5, 210)} = 0.786$ ;  $p=0.561$ ), alternations ( $F_{(5, 210)} = 1.226$ ;  $p=0.299$ ), repetitions ( $F_{(5, 210)} = 1.798$ ;  $p=0.116$ ) (**Fig. 3A**), left turns ( $F_{(5, 210)} = 1.275$ ;  $p=0.277$ ) or right turns ( $F_{(5, 210)} = 1.275$ ;  $p=0.277$ ) (data not shown). Males and females showed 75% and 76.47% of non-biased responses in the FMP Y-maze task, respectively, suggesting no sex differences in laterality. Finally, in the Pavlovian fear conditioning protocol, no significant difference for males vs females was observed in the retention index ( $t_{(28)} = 1.022$ ;  $p = 0.3157$ ) (**Fig. 3B**).

## 4. Discussion

In this study we evaluated whether there was evidence of sex differences in two behavioral domains, through the analysis of four different protocols: novel tank diving test (locomotion and anxiety), light-dark test (fear and anxiety), FMP Y-maze task (short-term spatial memory) and Pavlovian fear conditioning task (fear-related memory). We showed, for the first time in zebrafish, that females show stronger anxiety-like responses, spending more time in the bottom of the tank in the novel tank diving task, and remaining more in the dark zone of the light-dark task. We did not find any evidence of sex differences in zebrafish in the learning and memory-related phenotypes. Collectively, our data confirm that it is critical to consider sex as a factor when working with zebrafish anxiety-like behavior. In addition, our data reveal that zebrafish could be an important vertebrate translational model to assess the biological basis of sex differences in anxiety behaviors.

Similar to rodents, zebrafish have a well-known behavioral response to novelty, where their initial preference is for sheltered areas such as the bottom of the tank in the novel tank test, or the dark side of the tank in the light-dark test. Within the first few minutes of being exposed to a new place, the preference typically dissipates as the fish becomes habituated to the environment (Serra *et al.*, 1999; Egan *et al.*, 2009; Kalueff *et al.*, 2013; Tran & Gerlai, 2013). Few studies have considered sex as a factor in the novel tank diving test. There are some exceptions, however, with some studies not reporting any significant sex differences (Singer *et al.*, 2016; Clayman *et al.*,



2017), and other studies suggesting that females have a decreased anxiety-related phenotype when compared to males (Volkova *et al.*, 2015; Ampatzis & Dermon, 2016). Several sex-related performance differences have been observed in the novel tank test, with female zebrafish that display high activity preferring the top portion of the tank, and low activity females preferring the lower area, but males did not exhibit these behavioral patterns (Tran & Gerlai, 2013). Here, we found that females spend a longer time spent in the bottom zone of the novel tank diving task and longer time spent in the dark zone of the light-dark test, which is consistent with the hypothesis that traits of “anxious” behavior might be sex-dependent. Although both tests measure anxiety-like phenotypes in zebrafish behavior, researchers have suggested that the two tests are complementary rather than interchangeable. For example, the responses observed in the novel tank are based on the diving response and the animal patterns of exploration into a new environment, meanwhile the light-dark test assesses the animal search for a ‘shelter’ in a new environment. Thus, the diving response and scototaxis (dark-preference) may reflect dissociable mechanisms of behavior and different motivational aspects (Maximino *et al.*, 2010b; Blaser & Rosemberg, 2012) which both suggested that zebrafish females have an increased anxiety-like phenotype.

Zebrafish is a relatively new animal model, and our present insufficient understanding of factors that may influence their behavioral responses can directly affect in the data output (Gerlai, 2019). For example, some of the studies mentioned above had a handling period prior to the anxiety test (Ampatzis & Dermon, 2016; Singer *et al.*, 2016), used different zebrafish populations (Tran & Gerlai, 2013; Clayman *et al.*, 2017) or different tank color (Volkova *et al.*, 2015). These factors could justify the discrepancies among studies and is important to emphasize that all standard guidelines in zebrafish husbandry were followed in this experiment. The use of blinding and randomized experimental design to avoid publication bias and only trained researchers conduct the experiment to avoid any increase of stress levels caused by handling were also a prioritization.

In general, there are several factors that could explain why females and males have different patterns in these tasks, including exploratory drive (Gioiosa *et al.*, 2007; Gagnon *et al.*, 2016), territoriality (Ah-King *et al.*, 2005; Spencer & Smith, 2005) and aggression (Tieger, 1980; Reddon & Hurd, 2008) or other behaviors associated to reproduction (Magurran & Maciás Garcia, 2005). Thus, further studies associating other behavioral patterns could help to elucidate the differences founds here. In addition, future studies may probe underlying mechanisms of sex differences in anxiety in zebrafish using pharmacological interventions. For example, it would be

useful to evaluate any differences in the impact of anxiolytics on behavioral performance. Studies have accounted for sex in their statistical analyses, but the effect sizes we have observed here would suggest that ceiling effects may have masked detectable differences.

Concerning learning and memory, the FMP Y-maze task is commonly used to assess short-term spatial memory in rodents (Nakagawa *et al.*, 2004) and has recently been adapted for zebrafish (Cleal & Parker, 2018). Adapting a framework that has previously been used for analyzing search and exploratory patterns in the T-maze (Gross *et al.*, 2011), it is possible to create tetragrams and observe the number of alternations (rlrl + lrlr) and repetitions (rrrr + llll). Both alternations and repetitions are not only linked with exploratory patterns but also involve seem to involve short-term spatial memory, as evidenced by reductions in alternations following developmental alcohol exposure (Cleal & Parker, 2018). During Pavlovian fear conditioning, organisms learn to predict aversive events and then its memory to aversive contexts can be measured (Rudy *et al.*, 2004; Manuel *et al.*, 2014; Manuel *et al.*, 2015; Brock *et al.*, 2017). Several studies have shown that females have differences in memory tasks. For example, in humans, females perform better than males on episodic memory tasks such as word recognition and recall (Zelinski *et al.*, 1993; Herlitz *et al.*, 1997), face and name recognition and recall (Larrabee & Crook, 1993; Herlitz *et al.*, 1997). Previous studies have found that zebrafish females and males that are housed in segregated groups have more persistent inhibitory avoidance memory than mixed housed (50:50 females: males) groups; however no overt sex differences were found (Reolon *et al.*, 2018). Here, we confirmed this lack of sex difference in learning/memory, not finding any evidence for sex differences in two zebrafish learning/memory responses. Importantly, in this study animals were pair-housed for 24 h prior the memory and cognition-related tests, as previous studies have shown that there are differences in the performance of pair-housed animals compared to those acclimatized in groups (Parker *et al.*, 2012). Although the use of animals in multiple behavioral task have been widely used in other translational species, there is no evidence of how the use of zebrafish in multiple tasks could affect their behavioral phenotypes. Thus, the use of zebrafish in multiple tasks and the lack of acclimatization prior to our pair-housing system could affect sex differences of zebrafish performance in the FMP Y-maze and Pavlovian fear conditioning task.

There are several reasons why sex differences may be observed across species. Behavioral sex differences may relate to differences in brain structure, neurochemistry, neuroendocrinology and neurobiology (Cahill, 2006; Cosgrove *et al.*, 2007; Solomon & Herman, 2009). For example,

in rodents there are robust sex differences in the magnitude of long-term potentiation responses and NMDA activation when presented with contextual conditioned stimuli (Maren et al., 1994), which may explain some different behavior patterns observed for females vs males. At odds with humans and rodents, we found that female zebrafish present high patterns of anxiety and no difference in learning and memory tasks. Although zebrafish neuroanatomical and neurochemical pathways are highly conserved with human brain, the overall organization of major brain components is topographically distinct from mammals (Rupp *et al.*, 1996; Ullmann *et al.*, 2010). Some notable differences in the relative size of brain structures (e.g. smaller cerebral hemispheres) (Tropepe & Sive, 2003) may have an impact on different patterns of behavior observed here.

In humans, sex differences are also critically affected by external factors such as different influences from environmental, cultural and social roles (Eagly, 2009; Davis *et al.*, 2015). Overall, these factors make the translational association of sex differences more complex when comparing different species such as rodents and zebrafish. Importantly, although it is difficult to translate the memory and anxiety-like behavior phenotype of zebrafish to humans fully, the differences may represent underlying neurobiological differences that could be studied in zebrafish. This might be important, as in humans, sex differences are often highly variable and conflicting. This high level of variation may be due to hormonal changes linked to the menstrual cycle (Toufexis *et al.*, 2006; Goldstein *et al.*, 2010). These hormonal patterns are observed in other mammals, but as zebrafish are oviparous they do not present such cycles (Spence *et al.*, 2008; Singleman & Holtzman, 2014).

## 5. Conclusion

In summary, zebrafish females presented increased anxiety-like behavior compared to males, in two distinct anxiety tasks. No sex-dependent responses in learning and memory were observed, either for short-spatial or fear-related, memory. Thus, our data strongly suggest that the anxiety levels of female vs male need to be taken into account when using zebrafish as a translational model, as it clearly represents an important variable in behavior analysis. Another important point is the evolutionary aspect of translational research. Humans and translational models have both differences and similarities in how sex influences behavior, and the study of common neurological pathways and genetic factors that link those differences may improve our knowledge about human conditions. Overall, although females present more anxious traits, the

motivational, neurological and genetic factors underlying different anxiety-like phenotypes in zebrafish male vs female still not known and more studies are necessary.

### **Conflict of interest**

The authors declare that no conflict of interest exists.

### **Data accessibility statement**

All raw data from this study are openly available in the University of Portsmouth Pure repository, at [researchportal.port.ac.uk/portal/](https://researchportal.port.ac.uk/portal/)

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### **Author contributions**

B.D.F., M.C. and M.O.P conceived and designed the research. B.D.F. and M.C. conducted the behavioral experiments. B.D.F. and M.O.P performed data analyses. B.D.F., M.C. and M.O.P wrote the manuscript.

### **Abbreviations**

CS: Conditioned stimulus

FMP: Free movement pattern

ITI: Inter-trial interval

NMDA: N-methyl-D-aspartate

S.E.M.: Standard error of the mean

US: Unconditioned stimulus

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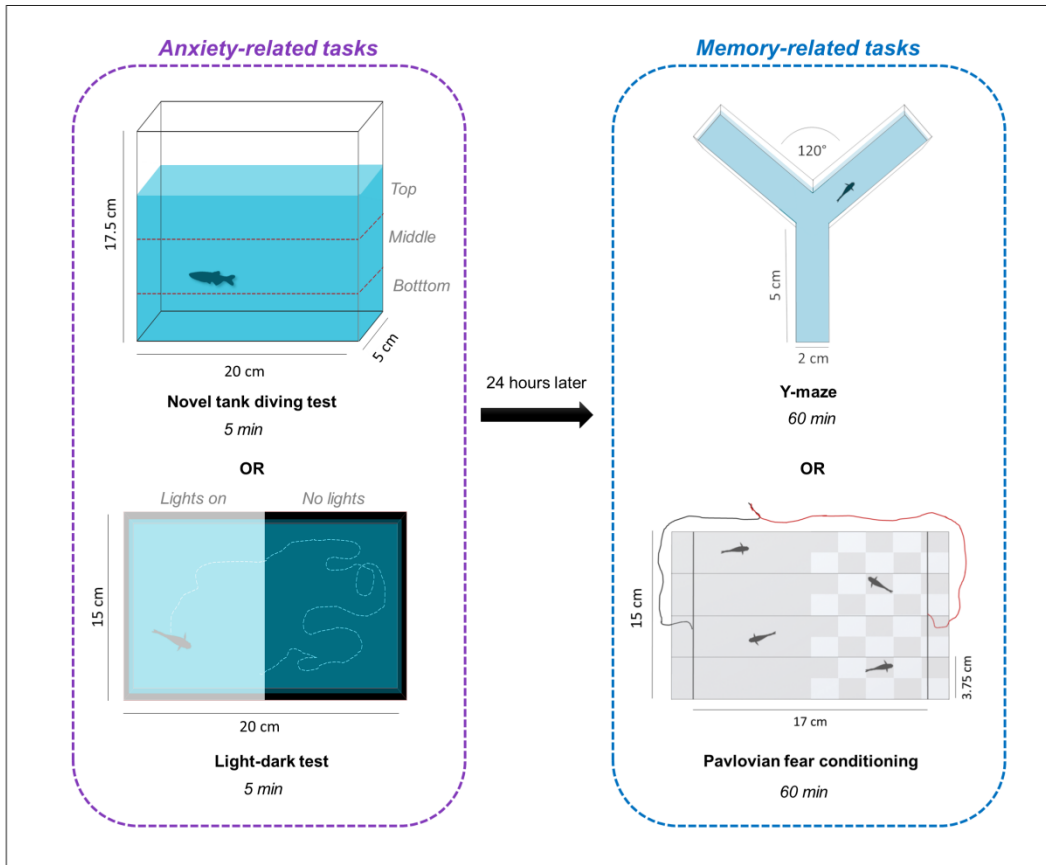
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### Figure Legends

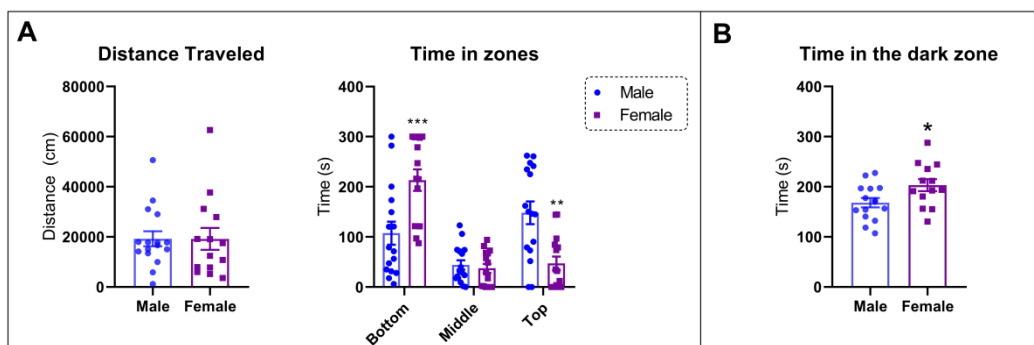
**Figure 1.** Schematic representation of the behavioral tasks used to assess sex differences in anxiety and memory of adult zebrafish.

**Figure 2.** Sex differences in anxiety-related tasks. **(A)** No alteration in the distance traveled was observed, but a significant increase time in bottom area and decreased time in top area was observed for females in the novel tank diving task ( $n = 16 - 17$  per group). **(B)** Time spent in the dark zone is significantly increased in females compared to males in the light-dark task ( $n = 15 - 17$  per group). Data were represented as mean  $\pm$  S.E.M. and analyzed by linear mixed models (time spent in zones) or Student's T-Test (distance traveled, and time spent in dark zone). Asterisks indicates statistical differences comparing females vs males (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ )

**Figure 3.** Effects of sex in learning and memory tasks. **(A)** No significant sex effect was observed for the total turns, repetitions and alternations in the spontaneous Y-maze task ( $n = 16 - 17$  per group). **(B)** Sex did not influence the performance in the Pavlovian fear conditioning ( $n = 15 - 17$  per group). Data were represented as mean  $\pm$  S.E.M. and analyzed by linear mixed models (total turns, repetitions and alternations) or Student's T-Test (retention index).

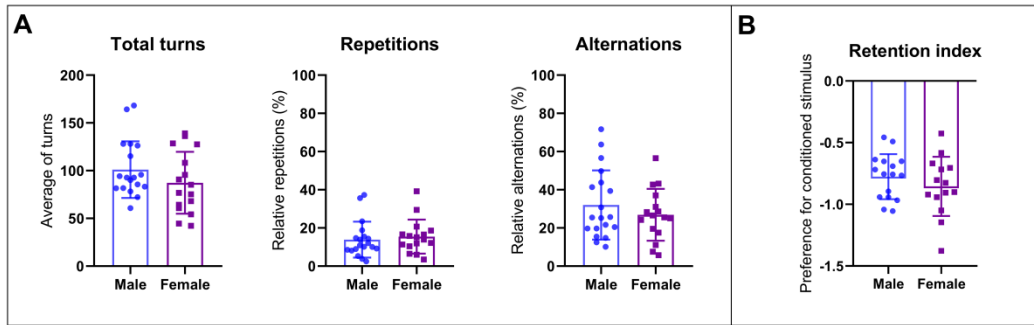


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