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Long-term behavioral alterations following embryonic alcohol exposure in three zebrafish populations

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ABSTRACT

Fetal alcohol exposure may lead to a condition known as fetal alcohol spectrum disorder (FASD), which comprises a set of consequences, including cognitive and behavioral impairments. Although zebrafish has been applied as a reliable model for studying FASD, there is no approach to the disorder's ontogeny and population differences. Here, we evaluated the behavioral outcomes of AB, Outbred (OB), and Tübingen (TU) zebrafish populations embryonically exposed to alcohol throughout the development to the adult stage. We exposed 24hpf eggs to 0 %, 0.5 %, or 1.0 % alcohol for 2 h. Fish were let grow and locomotor and anxiety-like behaviors were tested in a novel tank at larval - 6dpf, juvenile – 45dpf, and adult- 90dpf stages. At 6dpf, both AB and OB treated with 1.0 % alcohol showed hyperactivity, while 0.5 % and 1.0 % TU fish exhibited hypolocomotion. At 45dpf, AB and TU fish maintained the larval pattern of locomotion. At the adult stage – 90dpf, both AB and TU populations showed increased locomotor activity and anxiogenic responses, while the OB population did not show altered behavior. Our results show for the first time that zebrafish populations exhibit behavioral differences in response to embryonic alcohol exposure and that it varies along animals' ontogeny. AB fish showed the most consistent behavioral pattern through developmental stages, TU fish showed behavioral changes only in adulthood, and OB population showed high interindividual variability. These data reinforce that different populations of zebrafish are better adapted to translational studies, offering reliable results in contrast to domesticated OB populations obtained from farms, which exhibit more variable genomes.

1. Introduction

Alcohol is a teratogenic agent that affects the developing fetus's central nervous system, which is considered a major public health problem (Little et al., 2021). Although it varies between individuals, embryonic alcohol exposure usually leads to brain damage, growth problems, and behavioral challenges (Cadena et al., 2020). The condition is known as fetal alcohol spectrum disorder (FASD), which includes alcohol-related neurodevelopmental disorders (ARND) and the more severe outcomes named fetal alcohol syndrome (FAS) (May et al., 2014; Seguin and Gerlai, 2018).

FASD is characterized by varied levels of neural and behavioral alterations caused by alcohol exposure following alcohol concentration, exposure regime, and period of embryonic exposure (Baggio et al., 2018). Although medical and psychological treatments throughout the lifespan help mitigate the effects, there is no cure for FASD. The primary way to avoid the condition is abstinence from alcohol during the gestational phase. Alcohol exposure during the first trimester of pregnancy is particularly worrying because several processes of the neural system development arise during this phase, including the formation of the neural tube, neurogenesis, and migration of neurons (Chung et al., 2021; de Graaf-Peters and Hadders-Algra, 2006; Rice and Barone, 2000). However, around 10 % of pregnant woman consumes alcohol during pregnancy worldwide. The European region has a consumption rate much higher than the world rate, about 25 % (Popova et al., 2022). A meta-analysis conducted in 2017 reported an estimate that more than 1700 babies are born every day with FASD, which means that more than 630,000 each year worldwide are affected with this disorder (Lange et al., 2017).

While FAS usually includes morphological, cognitive, and behavioral

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alterations that favor diagnosis, mild cases included in the ARND are of a difficult diagnosis and frequently confused with other behavioral disorders (Mattson et al., 2019). Some studies show that individuals with FASD often present social skills difficulties during life, susceptibility to major depression, and impairment in cognitive abilities, including language and motor function (Lange et al., 2019). Besides that, individuals affected by FASD often present a tendency to develop alcohol and other drugs dependency in adulthood (Petrenko et al., 2014). However, there are individual and populational differences in response to alcohol exposure; some populations are more sensitive to the drug than others, while some are more tolerant than others (Leite-Ferreira et al., 2019; Mayfield et al., 2008).

Embryonic exposure to low alcohol concentration also results in varied phenotypes, including cases of ARND and individuals with no drug effects. While the genetic background may have a role in these differences, studies considering over-time changes in behavior in different populations may contribute to a better understanding of the embryonic alcohol effects and help to establish a more robust diagnostic chart, as metabolism and the effect of alcohol may be differences between racial/ethnic groups due to biological and genetic variations in populations (Wall et al., 2016).

In this sense, studies comparing different zebrafish populations, like Outbred (OB), Tübingen (TU), and AB, and may help a thorough understanding of the alcohol effects since they exhibit different behavioral and neurochemical responses to alcohol. For instance, the TU population seems to be more resistant, while animals from the AB population are more sensitive to alcohol effects (Agues-Barbosa et al., 2022; Loucks and Carvan, 2004; Mahabir et al., 2014; Pannia et al., 2014). AB populations also show superexpression of genes that code neurotransmitter receptors, such as dopamine, GABA, and glutamate, compared to fish with undefined genetic backgrounds (Pan et al., 2012).

The zebrafish is a well-established animal model for research in general disorders related to alcohol, both in adults and larval stages (Agues-Barbosa et al., 2022; Baggio et al., 2018; Leite-Ferreira et al., 2019; Pinheiro-da-Silva et al., 2021; Pinheiro-Da-Silva et al., 2020; Suresh et al., 2021; Tsang et al., 2019). We hypothesized that behavioral differences presented by different populations and at different developmental phases are related to population features, such as genetic background. Thus, the present study investigated the embryonic effects of alcohol observable through the ontogenetic development in zebrafish from three populations: TU, OB, and AB.

2. Methods

2.2. Animals housing

Adult (6 months) zebrafish from TU, OB, and AB populations were kept in acrylic tanks in an automatized rack system (ZebTEC Active Blue Stand Alone Tecniplast®) with recirculating water-controlled conditions: 28 °C; pH 7.0; oxygen at ~6 mg/L; total ammonia at < 0.01 mg/L; and conductivity of 1600 μS/cm. The room photoperiod was set at 14L/10D (light/dark). Animals were fed twice a day with nauplii of *Artemia sp.* (Artemia salina do RN®, Brazil) and flake food (Alcon Basic® 60 % protein; 15 % fat). OB fish were purchased from a local farm (Natal, Brazil), AB fish were donated from Pontifícia Universidade Católica do Rio Grande do Sul (PUC Rio Grande do Sul, Brazil), and TU fish were donated from University of Toronto (Mississauga, Canada). Although all the populations used herein are technically considered as Wild-type (WT) lines (i.e., lines with no defined mutations (Trevarrow and Robison, 2004)), We designate as OB fish, a genetically heterogeneous population with unknown genetic background, obtained from a local fish farm (Agues-Barbosa et al., 2022).

To test the effects of embryonic alcohol exposure on zebrafish, we paired adult breeding fish (>6 months old; in the ratio of two females and three males, OB, TU, and AB populations) for breeding purposes according to Spence et al. (2008). In total, at least four breeding tanks were set up for each studied population (2 females and 3 males/tank). On the day before fertilization, we transferred fish from the automatized system to reproduction tanks (22 cm × 13 cm × 12 cm - Beach Style Design - Tecniplast®). On the next morning between 60 and 120 min after the lights turn on (7 am), the eggs were collected and transferred to Petri dishes (6 cm) containing system water. At 24hpf (hours-post fertilization) eggs were treated as described below.

Around 50 eggs were then maintained in plastic trays (30. × 22. × 7.5 cm) filled with 1 L system water up to 5 dpf (days post-fertilization) and were fed with Paramecium until the 10th dpf. From 10–13 dpf larvae were fed with a mix of Paramecium and powdered food (Alcon Alevinos® 44 % protein; 5 % fat), and water level was raised to 2 L in each tray. From 13dpf until 45dpf, larvae were fed with powdered food and nauplii of *Artemia sp.*, and water levels was kept 4 L. At 45dpf fish were transferred to an automatized rack system (ZebTEC Active Blue Stand Alone - Tecniplast®) and fed with nauplii of *Artemia sp.* (Artemia salina do RN®, Brazil) and flake food (Alcon Basic® 60 % protein; 15 % fat).

2.3. Embryonic exposure to alcohol

Eggs collected from the breeding tanks of each zebrafish population were left in an incubator to complete 24 hpf, when eggs were separated into three experimental groups for alcohol exposure. The control group received 0.0 % alcohol (n = 50 for each population), the low-to-moderate group received 0.5 % alcohol (n = 50 for each population) and the moderateto-high group received 1.0 % alcohol (n = 50 for each population). To achieve desired concentration, alcohol (99.8 % absolute ethyl alcohol, Dinâmica, Química contemporânea Ltda. Brazil) was diluted in system water to 1.0 %. Eggs were exposed to alcohol for 2 h, following the protocol established by Pinheiro-da-Silva et al., (2020) Then, eggs were washed twice in system water and transferred to plastic trays.

2.4. Behavioral tests

Embryos exposed to alcohol were let grow and tested at three ontogenetic windows: 6, 45 and 90 dpf. We performed a novel tank test at the 3 ontogenetic phases (Fig. 1).

6dpf: larvae (AB n = 30; OB n = 34; TU n = 33) were individually transferred to a Petri dish (6 cm diameter) containing system water and behavior was recorded for 10 min using a web cam (*WebCam Logitech C525 HD*) positioned 1 m above the dish. Larvae were then returned to their housing conditions. We analyzed average swimming speed, total distance traveled, time stopped, time in the edge of the dish, and the distance from the edge. We considered as time in the edge when larvae remained up to 0.5 cm from the edge of the dish.

45dpf: Juvenile fish (AB n = 34; OB n = 40; TU n = 33) were individually transferred to acrylic tanks (12 × 10 × 4 cm) containing system water and behavior was recorded for 10 min with a camera positioned 1 m away and in front of the tank. Fish were then returned to their housing conditions. We analyzed average swimming speed, total distance traveled, time stopped and distance from the bottom of the tank.

90dpf: adult fish (AB n = 33; OB n = 31; TU n = 33) were individually placed into tanks (20 × 15 × 10 cm) containing system water and the behavior was recorded for 10 min as described for juvenile fish. Fish were then returned to their housing conditions. We analyzed the same parameters of the juvenile stage.

2.5. Data analysis

Behavioral records were analyzed using the tracking software Zebtrack/UFRN developed in MATLAB (R2015a; MathWorks, Natick, MA). Data were analyzed in software GraphPad. We tested each variable for normality and homoscedasticity using Shapiro-Wilk and Levene Test, respectively. Results showed that some variables were not

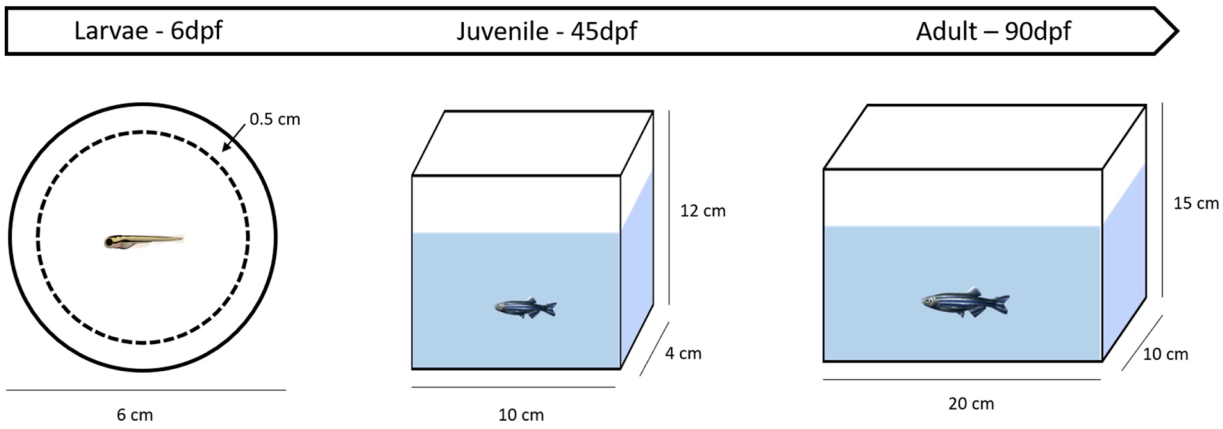

Fig. 1. Schematic view of the apparatus used along with ontogeny to evaluate behavioral endpoints in zebrafish. The arrow indicates the edge zone, set 0.5 cm from the border of the Petri dish.

homoscedastic. Thus, we carried out a Kruskal-Wallis or a One-Way ANOVA test when appropriated, followed by Dunn's post hoc or Student Newman Keuls – SNK test, respectively. Results were considered as significant when $\alpha < 0.05$. The p-value was adjusted in post hoc analysis.

3. Results

We performed a novel tank test to evaluate whether embryonic alcohol exposure affects the behavior of zebrafish throughout ontogeny in three zebrafish populations.

3.1. Behavioral response at the larval stage - 6dpf

The three zebrafish populations larvae differently responded to embryonic alcohol exposure. Larvae from both AB and OB populations exhibited hyperactivity, while TU larvae showed the opposite pattern of locomotor behavior (Fig. 2). In terms of average speed, One-Way ANOVA and Kruskal-Wallis indicated statistical significance between alcohol treatments (**AB**: $F_{(2,27)}= 4.22$; $p = 0.02$; **OB**: $F_{(2,31)}= 4.14$; $p = 0.02$, and **TU**: $H = 23.55$ df= 2; $p < 0.0001$). SNK Post hoc analysis revealed that hyperactivity appeared in AB larvae treated with 1.0 % alcohol ($p < 0.05$), while the OB population showed hyperactivity in both 0.5 % and 1.0 % groups ($p < 0.05$). For TU larvae, Dunn's test showed hypoactivity in both 0.5 % and 1.0 % groups compared to the control ($p < 0.05$). Regarding the total distance traveled, OneWay ANOVA indicated statistical significance for AB population ($F_{(2,27)}= 4.17$; $p = 0.02$) and OB population ($F_{(2,31)}= 3.7$; $p = 0.03$), and Kruskal-Wallis teste showed significance for TU population ($H = 22.83$; df=2; $p < 0.0001$).

SNK post hoc analysis revealed only 1.0 % treated AB larvae presented higher distance traveled in comparison to control ($p < 0.05$), in contrast to OB population, in which both 0.5 % and 1.0 % groups presented increased distance traveled compared to control ($p < 0.05$). Dunn's test showed that TU larvae treated with 0.5 % and 1.0 % alcohol presented decreased distance traveled compared to control ($p < 0.05$).

In terms of anxiety-like response, we measured larvae thigmotaxis through the time spent at the edge of the dish and the distance from the center of the dish (Fig. 3). Results showed that embryonic alcohol exposure appears to alter the anxiety-like responses in 6dpf larvae. One-Way ANOVA and Kruskal-Wallis tests showed statistical significance in the time spent at the edge of the dish for (**AB** ($F_{(2,27)} = 3.81$; $p = 0.034$), **OB** ($H = 15.90$; $p = 0.0004$), and **TU** ($H = 18.03$; $df = 2$; $p < 0.0001$) populations). SNK posthoc analysis showed that AB larvae treated with 0.5 % and 1.0 % alcohol and OB larvae treated with 1.0 % alcohol spent less time at the edge of the dish ($p < 0.05$). Dunn's test indicated that TU

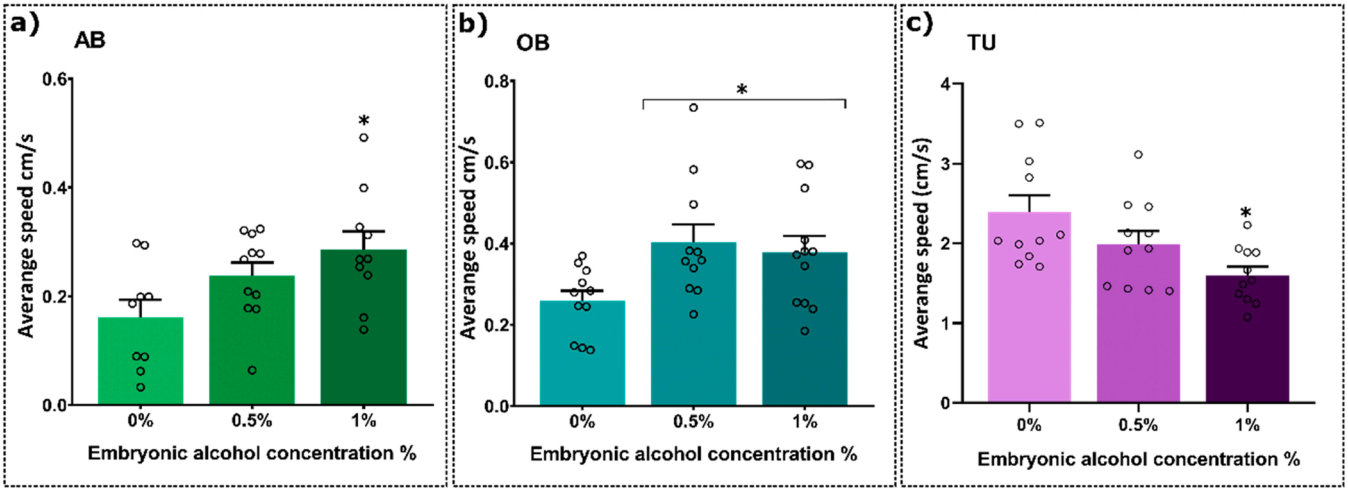

Fig. 2. Locomotor responses of three zebrafish populations (AB, OB, and TU) exposed to embryonic alcohol treatments – 0 %, 0.5 %, and 1.0 % (see methods for more details). Larvae were tested at 6dpf in the novel tank test. Fish behavior was recorded for 10 min, and each circle represents an animal. Average speed a) AB, b) OB, and c) TU were analyzed. Mean and SEM are represented. We compared fish behavior in relation to alcohol treatments of each strain using One-Way ANOVA or Kruskal-Wallis. Asterisks indicate statistical significance compared to the 0 % alcohol control.

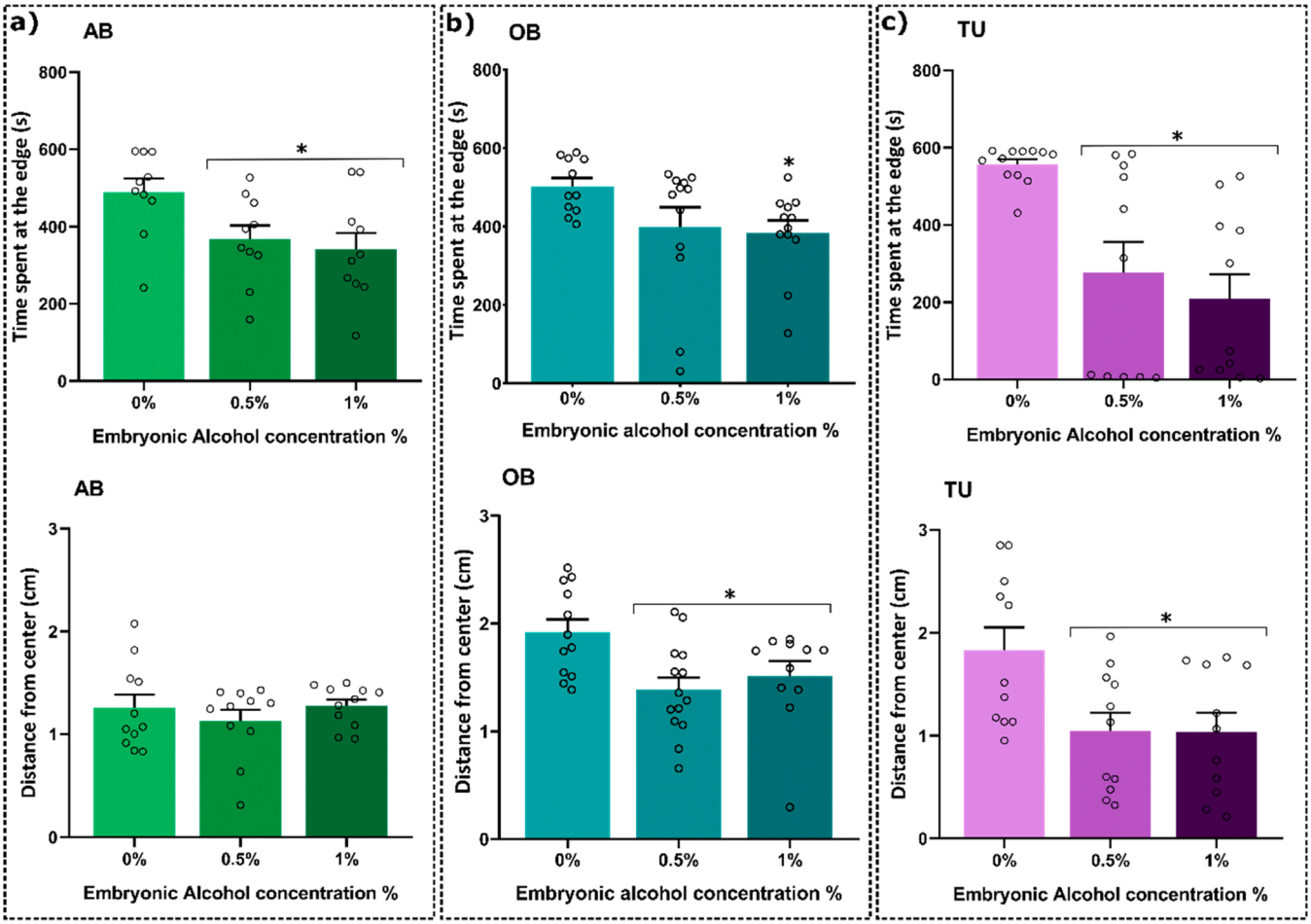

Fig. 3. Anxiety-like responses of three zebrafish populations a) AB, b) OB, and c) TU exposed to embryonic alcohol treatments 0 %, 0.5 %, and 1.0 %. Larvae were tested at 6dpf on novel tank test, fish behavior was recorded for 10 min. The time spent at the edge of the dish and the distance from the center of the dish were analyzed. We compared fish behavior of alcohol treatments from each strain through One-Way ANOVA or Kruskal-Wallis, with mean and SEM is represented. Asterisks indicate statistical significance compared to the 0 % alcohol control, $p < 0.05$.

larvae treated with 0.5 % and 1.0 % alcohol decreased time spent at the edge ($p < 0.05$).

For distance from the center of the dish, One-Way ANOVA showed no statistically significant results for AB population ($F_{(2,27)} = 0.52$; $p = 0.6$), but indicated statistical significance for TU and OB populations (OB: $F_{(2,31)} = 6.62$; $p = 0.0037$, and TU: $F_{(2,32)} = 4.41$; $p = 0.02$). SNK

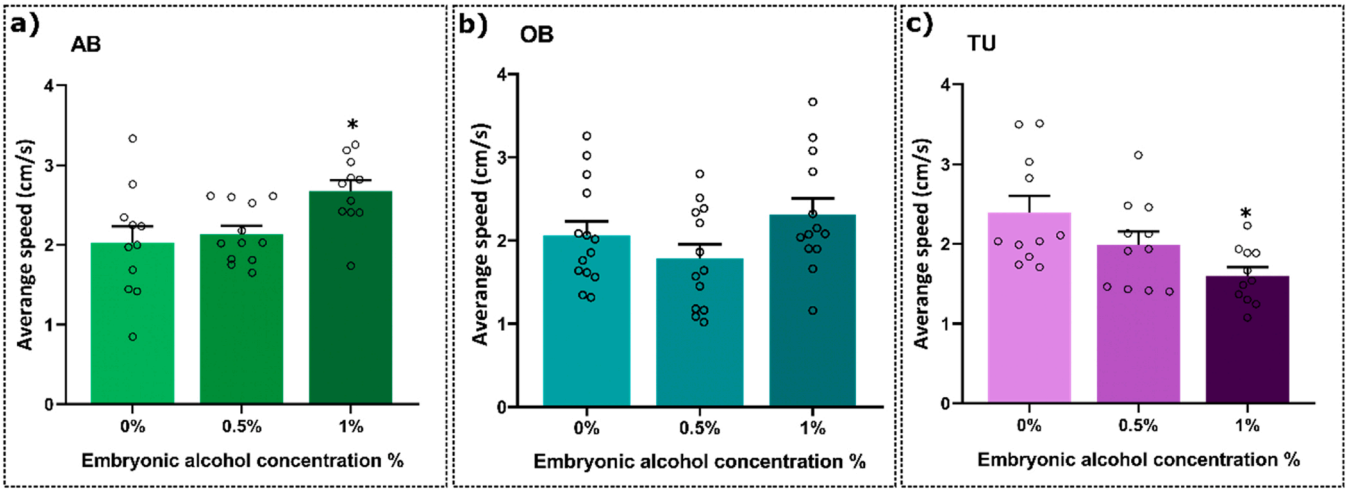

Fig. 4. Locomotor responses of three zebrafish populations (AB, OB, and TU) exposed to embryonic alcohol treatments – 0 %, 0.5 %, and 1.0 % (see methods for more details). Larvae were tested at 45dpf in the novel tank test. Fish behavior was recorded for 10 min, and each circle represents an animal. Average speed **a)** AB, **b)** OB, and **c)** TU were analyzed. Mean, and SEM are represented. We compared fish behavior in relation to alcohol treatments of each strain using One-Way ANOVA or Kruskal-Wallis. Asterisks indicate statistical significance compared to the 0 % alcohol control.

post hoc analysis showed that larvae from both populations exposed to 0.5 % and 1.0 % alcohol remained farer from the edge of the dish compared to control ($p < 0.05$). These results suggest that both 0.5 % and 1.0 % alcohol exposure during the embryonic stage caused anxiolytic effect in zebrafish larvae since animals spent significantly less amount of time at the edge of the dish and more time closer to the center of the dish compared to the control larvae.

3.2. Behavioral responses at the juvenile stage - 45dpf

In terms of locomotion (Fig. 4), AB and TU fish showed a response consistent with that observed in the larval phase. Regarding mean speed, the One-Way ANOVA test showed statistical significance between AB juveniles treated with 0.0 % and 1.0 % alcohol (increased speed observed for 1.0 % AB: $F_{(2.31)} = 5.201$; $p = 0.0113$), similar to the findings for the larval stage. One-Way ANOVA indicated statistical significance for TU juvenile fish ($F_{(2,30)} = 5.7$; $p = 0.008$), and the SNK post hoc test revealed that only juveniles exposed to 1.0 % alcohol exhibited hypolocomotion compared to the control ($p < 0.05$). For OB juvenile fish, One-Way ANOVA showed no statistical significance ($F_{(2,37)} = 2.21$; $p = 0.12$). The total distance traveled presented parallel results for the three populations. The One-Way ANOVA test showed that AB juvenile fish exposed to 1.0 % alcohol exhibited higher distance traveled than the control ($F_{(2,\ 31)} = 5.405$; $p = 0.009$), reinforcing the hyperactivity. Kruskal-Wallis test indicated statistical significance for TU juvenile fish ($H = 7.27$; $p = 0.03$) and Dunn's post hoc showed that TU treated with 1.0 % alcohol exhibited hypolocomotion compared to control ($p = 0.04$). The total distance traveled for OB juvenile fish was not statistically significant ($F_{(2,37)} = 2.82$; $p = 0.07$).

Given anxiety-like responses (Fig. 5), the outcomes obtained in the experiments showed similar behavioral pattern for both AB and TU populations. Concerning freezing, no statistical significance was obtained by the One-Way ANOVA test (AB ($F_{(2,\ 32)} = 2.26$; $p = 0.12$), and TU $F_{(2.30)} = 0.58$; $p = 0.57$) juvenile populations. However, Kruskal-Wallis analysis showed statistical significance for OB population ($H = 12.92$; $df = 2$; $p = 0.001$) and the Dunn's post hoc test showed that OB fish exposed to 0.5 % alcohol exhibited higher freezing than the control and 1.0 % groups ($p < 0.05$). For distance from the bottom of the tank, both AB and TU juvenile fish showed no statistical significance within groups (One-Way ANOVA, AB: $F_{(2,\ 32)} = 0.54$; $p = 0.58$), and TU: $F_{(2,29)} = 0.76$; $p = 0.47$). One-Way ANOVA indicated statistical significance for OB population ($F_{(2,36)} = 7.89$; $p = 0.001$). SNK post hoc test revealed that juvenile fish exposed to 0.5 % alcohol kept closer to the bottom of the tank (i.e., increased anxiety) compared to control group.

3.3. Behavioral response at adult stage – 90dpf

The motor response, average speed is presented in Fig. 6. Adult fish behavior was consistent with results found at the juvenile stage for the three populations. Statistical significance was observed by the One-Way ANOVA test in the average speed for AB fish ($_{F(2,30)} = 4.07$; $p = 0.02$) and TU ($F_{(2,30)} = 11.42$; $p = 0.0002$). Post hoc analysis showed that group AB fish with 1.0 % embryonic alcohol exposure showed faster swimming than the 0 % alcohol control. The post hoc analysis of the TU

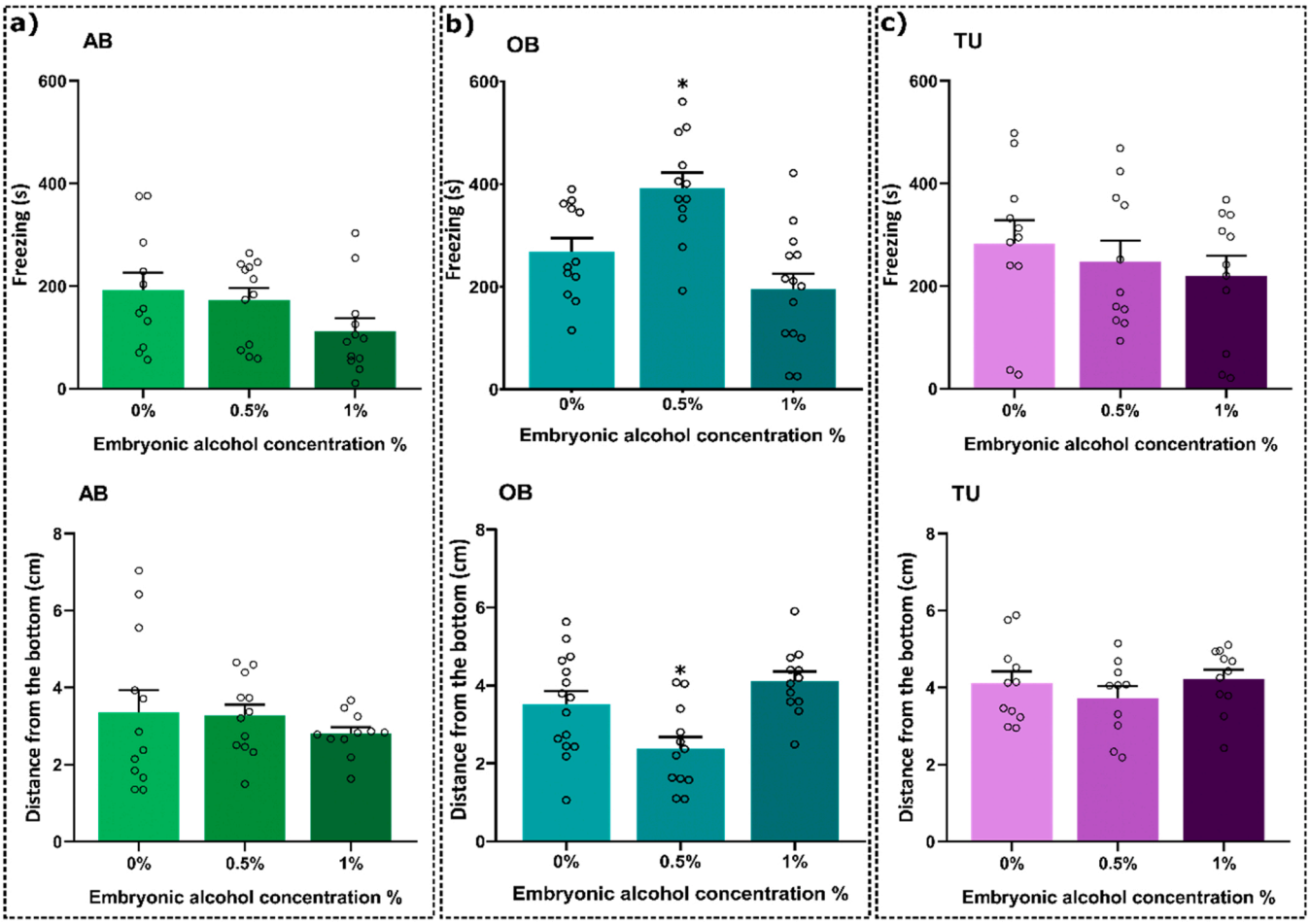

Fig. 5. Anxiety-like responses of three zebrafish populations a) AB, b) OB, and c) TU exposed to embryonic alcohol treatments – 0.0 %, 0.5 % and 1.0 % (see methods for more details). Juvenile were tested at 45dpf on novel tank test. Fish behavior were recorded for 10 min. Each circle represents one animal. Freezing, and distance from the bottom of the tank were analyzed. Mean and SEM are represented. Asterisks indicate statistical significance compared to the 0 % alcohol control, $p < 0.05$.

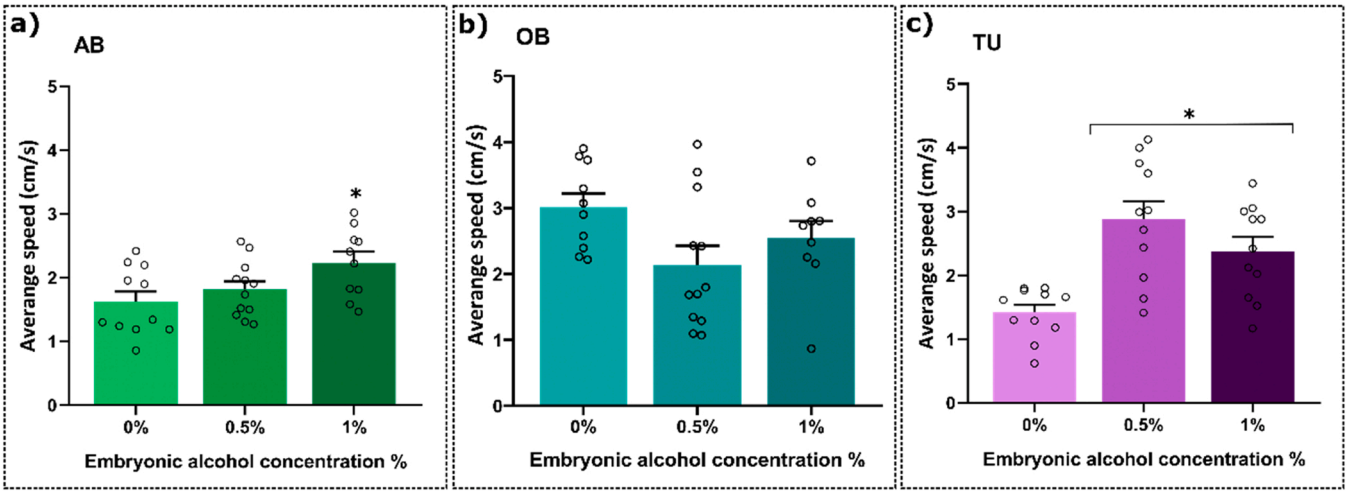

Fig. 6. Locomotor responses of three adult's zebrafish populations (AB, OB, and TU) exposed to embryonic alcohol treatments – 0 %, 0.5 %, and 1.0 %. Larvae were tested at 90dpf in the novel tank test. Fish behavior was recorded for 10 min, and each circle represents an animal. Average speed a) AB, b) OB, and c) TU were analyzed. Mean, and SEM are represented. We compared fish behavior in relation to alcohol treatments of each strain using One-Way ANOVA. Asterisks indicate statistical significance compared to the 0 % alcohol control.

fish treated with 0.5 % and 1.0 % alcohol showed that both groups showed faster swimming than the control ($p < 0.05$). One-Way ANOVA indicated no statistical significance for OB population ($F_{(2,28)} = 2.98$; $p = 0.07$). Concerning total distance traveled, results were similar to those from average speed. One-Way ANOVA showed statistical significance for AB fish ($F_{(2,30)} = 6.68$; $p = 0.004$), and One-Way ANOVA

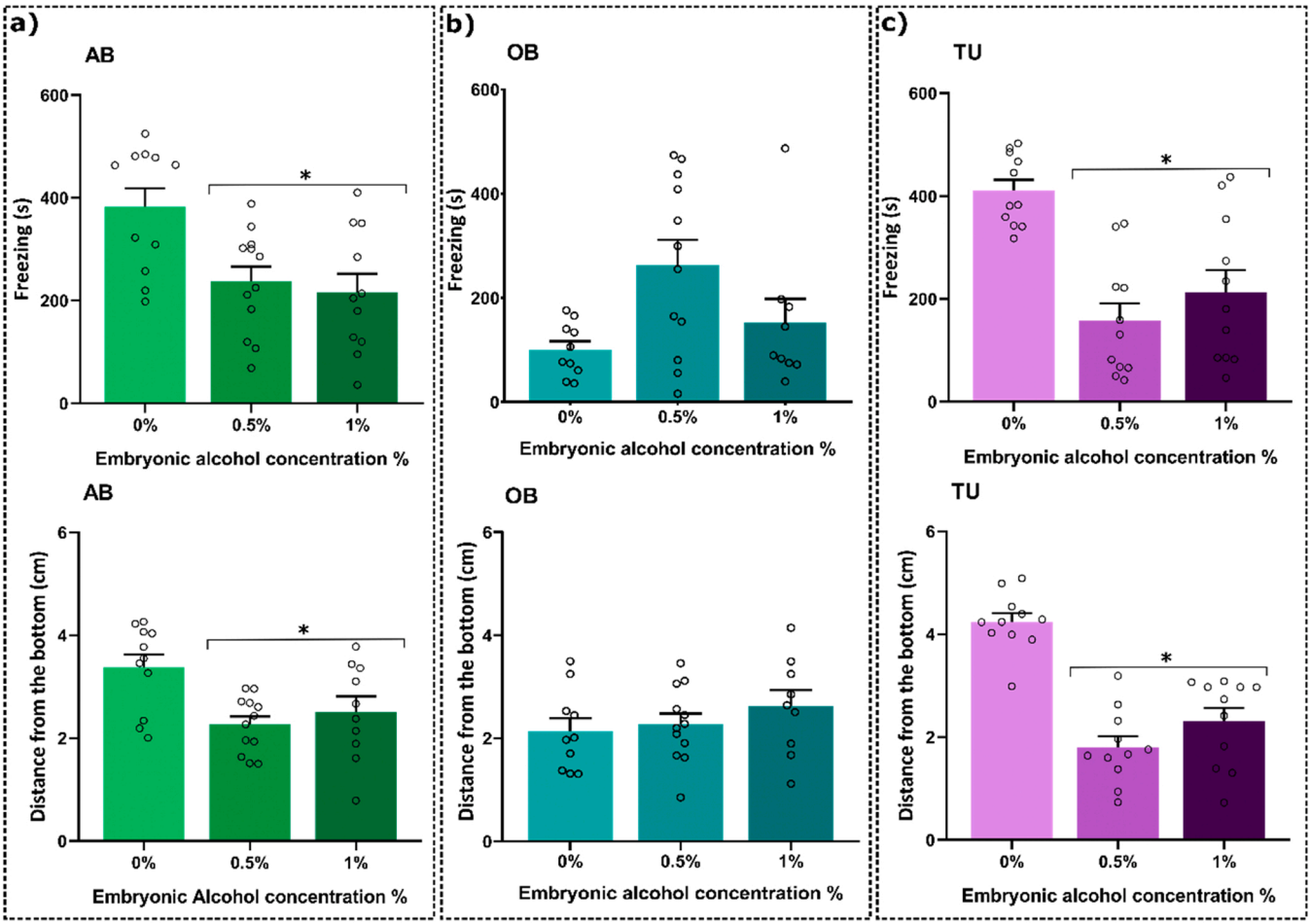

Fig. 7. Anxiety-like responses of three adult's zebrafish populations a) AB, b) OB, and c) TU exposed to embryonic alcohol treatments – 0.0 %, 0.5 % and 1.0 %. Adults were tested at 90dpf on novel tank test. Fish behavior were recorded for 10 min. Each circle represents one animal. Freezing, and distance from the bottom of the tank were analyzed. Mean and SEM are represented. Asterisks indicate statistical significance compared to the 0 % alcohol control, $p < 0.05$.

indicated statistical significance for TU fish ($F_{(2,30)}$= 11.48; p = 0.0002) and but no statistical significance for OB fish ($F_{(2,28)}$ = 2.98; p = 0.07). The analysis of the post hoc SNK test showed that the TU and AB groups (0.5 % and 1.0 %) increased locomotion concerning the control ($p < 0.05$), indicating that embryonic exposure to alcohol caused hyperlocomotion in fish adults in both groups.

In terms of anxiety-like responses, freezing and distance from the bottom of the tank are shown in Fig. 7. The behavioral pattern exhibited in AB and TU populations were similar in both measures. Statistical significance was obtained for freezing behavior in AB (One-Way ANOVA $F_{(2,31)} = 6.94$; p = 0.003) and TU populations (One-Way ANOVA $F_{(2,30)} = 15.63$; $p < 0.0001$). Post hoc analysis regarding TU, and AB fish showed that 0.5 % and 1.0 % alcohol groups spent less time in freezing compared to the control ($p < 0.05$). Kruskal-Wallis test showed no statistical significance for freezing in OB fish (H = 5.12; p = 0.08). For distance from the bottom of the tank, statistical significance was observed for AB and TU fish (AB: $F_{(2,30)} = 6.37$; p = 0.004; TU: $F_{(2,30)} = 32.90$; $p < 0.0001$). For TU, and AB fish, SNK post hoc analysis indicated increased time closer to the bottom of the tank for 0.5 % and 1.0 % alcohol groups ($p < 0.05$). For OB fish, One-Way ANOVA indicated no statistical significance in the distance from the bottom of the tank ($F_{(2,28)} = 0.87$; p = 0.43).

4. Discussion

We observed that embryonic alcohol exposure led to different locomotor and anxiety-like behaviors depending on the zebrafish's population and ontogenetic stage. From the three populations studied (AB, TU, and OB), AB presents consistent behavioral alterations throughout ontogeny, maintaining increased locomotion and reduced anxiety along the developmental stages (6, 45, and 90 dpf). TU population showed varied locomotion responses across time: hypolocomotion observed at 6 and 45 dpf became hyperlocomotion in adulthood, while anxiety reduced after alcohol treatment. For the OB population, the behavioral response was the most inconsistent, as locomotion increased only at the larval stage and anxiety-like responses that were reduced in larvae turned to an anxiogenic behavior in juveniles but not adults. It is the first study to demonstrate long-term behavioral changes in zebrafish populations embryonically exposed to alcohol and tested through ontogenetic stages.

As observed in other studies, alcohol exposure causes different effects on different populations, both when alcohol exposure occurs in adulthood (Agues-Barbosa et al., 2022; Gerlai et al., 2008; Pannia et al., 2014) and when embryos are treated with alcohol and behavioral manifestations are observed later on (Abozaid et al., 2020; Azimian Zavareh et al., 2022; Loucks and Carvan, 2004; Mahabir et al., 2014). In a study by Agues-Barbosa et al. (2022), chronic alcohol exposure applied to a OB population caused the most inconsistent response, especially regarding anxiety-like behavior. In the present study, the effect of embryonic alcohol exposure was evaluated at three developmental phases. Although the differences in behavioral patterns between populations are not apparent at 6dpf (Fig. 2, and Fig. 3), at later stages, as in juveniles (Fig. 4, and Fig. 5) and adults (Fig. 6, and Fig. 7), the behavioral patterns are in agreement to other studies: OB fish presenting the most divergent response when compared to AB and TU strain. Noteworthy, all animals were bred and kept under the same environmental conditions; thus, behavioral variability must result from differences in genetic background.

It is known that fetal alcohol spectrum disorder – FASD causes cell death (Alsakran and Kudoh, 2021), GABAergic system disruption, and adult CNS impairments (Cole et al., 2012). In this study, we exposed 24hpf zebrafish embryos to low and moderate alcohol concentrations (0.5 % and 1.0 %, respectively) to simulate FASD (Fernandes and Gerlai, 2009). Previous studies showed that alcohol concentrations used here do not produce severe malformations or physical abnormalities and do not affect the survival rate of zebrafish (Fernandes and Gerlai, 2009; Paul et al., 2020; Seguin and Gerlai, 2018), but it impairs social behavior (Fernandes et al., 2018, 2015; Fernandes and Gerlai, 2009; Parker et al., 2014), anxiety-like behavior (Abozaid et al., 2020; Baggio et al., 2018; Pinheiro-Da-Silva et al., 2020), learning and memory (Fernandes et al., 2014; Pinheiro-da-Silva et al., 2021), and locomotor responses (Abozaid et al., 2020; Ramlan et al., 2017). Even though the studies cited above used different zebrafish populations, here we compared three populations subjected to the exact condition of alcohol exposure and tested at the same stages in the same behavioral protocol.

We assessed the effects of embryonic alcohol exposure through the novel tank test applied at three ontogenetic stages. The test allows the analysis of both locomotor responses and anxiety-like behavior (Maximino et al., 2010). In locomotion, the increased swimming speed and distance traveled (i.e. hyperlocomotion) observed in AB and OB fish are associated with hyperactivity, a known consequence of fetal alcohol exposure. At the larval stage, Ramlan et al. (2017) also found that OB zebrafish embryonically exposed to alcohol exhibit increased locomotion, while Abozaid et al. (2020) found similar results for AB zebrafish but no effects in a OB population. The high variability observed in OB populations reported in different studies is associated with rearing conditions and genetic background, as detected in our study. However, for TU larvae, we observed the opposite pattern: 6dpf larvae showed decreased locomotion (i.e. hypocolomotion). In another study, we observed that some proteins related to locomotor response, such as acetylcholinesterase (AChE) and tryptophan hydroxylase (TH1) show different levels of expression in AB and TU fish chronically treated with alcohol. The increased expression of TH1 in AB, while TU is unaltered, and the reduced expression of AChE in TU, while AB is unaltered, are related to differences in locomotor response (Agues-Barbosa et al., 2022). These populations present several differences related to gene expression due to alcohol exposure, including increased BDNF expression in TU, what may affect how the organisms deal with toxic agents exposure (alcohol), and how it affects the individual's performance later on (Agues-Barbosa et al., 2022).

Regarding anxiety-like responses, all populations tested here (AB, TU and OB) exhibited anxiolytic responses at some stage: FASD larvae spent less time at the edge of the dish and kept closer to the center of the dish compared to the control (Fig. 3). Our results follow others that tested AB and OB larvae treated with alcohol and showed decreased anxiety at 6pdf (Ramlan et al., 2017), 7dpf (Abozaid et al., 2020), and 9dpf (Baiamonte et al., 2016). Alcohol exposure affects how animals perceive environmental threats and deal with them (Pinheiro-da-Silva et al., 2021), increasing individuals' risk exposure. Embryo alcohol exposure affected zebrafish larvae from the three populations tested. Thus, our results support the use of zebrafish as an indicator of early-onset symptoms of fetal alcohol exposure (Pinheiro-da-Silva et al., 2021; Pinheiro-Da-Silva et al., 2020).

Along with ontogenetic development, the motor response profile observed in the AB population remained consistent (hyperlocomotion in the alcohol-treated groups). In contrast, TU zebrafish presented the opposite response at 45dpf, and OB zebrafish did not show any effect in the juvenile and adult stages (Fig. 4). As discussed earlier, the hyperactivity response in AB alcohol-treated groups was expected since it is a prevalent symptom of FASD. This phenomenon is caused by the alcohol effects on several neurotransmitter systems associated with motor control, such as GABAergic and dopaminergic (Rico et al., 2011).

Other authors also observed hyperactivity in response to embryonic alcohol exposure in 60dpf zebrafish (Bailey et al., 2015; Burton et al., 2017). In contrast, the motor response observed in juvenile alcohol-treated TU suggests a hypoactivity profile in this population. Once again, the behavioral response observed for TU differs from other populations and could be related to the differential effects of alcohol on gene expression (Agues-Barbosa et al., 2022) and brain physiology (i.e., dopaminergic system – see Mahabir et al., 2014) in this population. Regarding OB fish, the lack of robustness observed in this genetically heterogeneous population suggests its high degree of polymorphisms.

Moreover, similar to other studies (Bailey et al., 2015; Burton et al., 2017), juvenile AB and TU zebrafish did not show anxiogenic responses following embryonic alcohol exposure (Fig. 5). Juvenile zebrafish has been validated as a pharmacological and behavioral model for assessing anxiety-like responses (Varga et al., 2018). However, it is usually tested in other protocols but in the novel tank test. The novel tank is a reliable test to evaluate anxiety-like behavior. It is an adapted version of the Open Field Test, a commonly used protocol to obtain behavioral data related to animal locomotion and anxiety. The novel tank helps to identify the pharmacological effects of drugs applied to various species as rodents (La-Vu et al., 2020; Seibenhener and Wooten, 2015), primates (Cagni et al., 2012), and fish (Baker et al., 2018; Maximino et al., 2010; Yoshida, 2022). Despite this, the absence of anxiety-like behavior found in 45dpf zebrafish is related to alcohol's modulation of the neurotransmitter systems during CNS development. Although Mahabir et al. (2014) found that AB and TU zebrafish embryonically exposed to alcohol present altered levels of serotonin and dopamine at 40dpf, further analysis must be conducted, especially considering other neurotransmitters, such as GABA and glutamate, and the effects on genes related to these products. We suggest that future studies must validate the anxiety-like responses in zebrafish at different ages using classical protocols, such as dark-light avoidance (i.e., light/dark) and novel tank test, and correlate the behavioral responses to neurophysiological parameters.

Additionally, we analyzed the behavior of adult FASD fish – at 90dpf (Fig. 6, and Fig. 7). The results obtained agree with previous studies using the same populations (Agues-Barbosa et al., 2022). We observed hyperactivity in both AB and TU fish but no changes related to OB locomotor behavior. Although the behavioral pattern of TU fish was completely altered from larvae to the adult stage, changes intra and inter-population in locomotor patterns along with ontogeny were already reported (Lange et al., 2013).

Most of the studies approaching FASD in zebrafish evaluate its effects in adults. Moreover, studies often evaluate the socially disrupted response and seldom report locomotor alterations (Buske and Gerlai, 2011; Fernandes et al., 2018; Fernandes and Gerlai, 2009). Here we observed that motor response (i.e., average speed and total distance traveled) suffered consistent change from the larval stage, especially in AB and TU fish. This response seems to be related to early disruptions in the dopaminergic system, a phenomenon already reported in adult zebrafish embryonically exposed to alcohol (Buske and Gerlai, 2011; Facciol and Gerlai, 2020; Mahabir et al., 2014).

AB and TU fish treated with alcohol expressed anxiogenic responses, indicated by the distance from the bottom line (Fig. 7), while OB fish did not change behavior. Freezing behavior and distance from the bottom are data used to assess behavior with an anxious profile. The adult AB matrix (Fig. 7a) showed reduced freezing after exposure to alcohol, which may indicate possible reduced anxiety, but the reduced distance from the background indicates higher anxiety.

Although data on the anxious profile of the freezing parameters and distance from the bottom show divergence, studies have reported that freezing time is often not the best indicator to assess the anxious profile of fish, as it is difficult to distinguish whether the individual's behavior was freezing, immobility or rest (Kalueff et al., 2013). The same authors report that the distance from the bottom is the most suitable parameter to assess the anxious profile in zebrafish. Therefore, during the adult phase, these fish from the AB population showed an anxiogenic profile. The increase in locomotion in AB fish exposed to alcohol also corroborates data from the measurement of the distance from the bottom, a likely increased anxiety.

Baggio et al. (2018) reported increased time in the bottom of the tank in adult AB zebrafish embryonically exposed to both 0.5 % and 1.0 % alcohol. For TU, Baiamonte et al. (2016) showed that 48hpf embryos exposed to alcohol exhibited anxiolytic response at the adult stage. These responses could be related to ontogenetic development differences in each population, which should be considered in future studies, or alcohol disruption to the serotoninergic system during CNS early development. For instance, TU fish embryonically exposed to alcohol produce altered mRNA levels of serotonin receptor - 5-HT1A (Parker et al., 2014), while FASD AB fish produced altered levels of serotonin and its metabolite (5-Hydroxyindoleacetic acid - 5HIAA) in adulthood (Buske and Gerlai, 2011; Mahabir et al., 2014). On the other hand, OB fish showed behavior similar to control fish, which could be related to a higher adaptative capacity to recover from alcohol exposure or to high variability between individuals that mask the response. Likewise, we observed in another study that both populations (TU and AB) modulate the tryptophan hydroxylase similarly and exhibited a comparable behavioral pattern (increased anxiety during withdrawal). In contrast, the OB population showed no significant response (Agues-Barbosa et al., 2022).

These results indicate that embryonic alcohol exposure affects CNS development until the late ontogenetic stages. More than that, the effects appear to be long-lasting until the elderly age (Fernandes et al., 2015; Harvey et al., 2019) since animals tested at two years old showed altered social response. For future studies, other ontogenetic stages should be considered and compared in terms of morphological development. Also, research approaching molecular analysis at each developmental stage should be considered to establish the association between behavior and genetic responses in zebrafish populations.

5. Conclusion

In summary, zebrafish populations exhibited different long-term behavioral patterns in response to embryonic alcohol exposure. While populations (AB and TU) presented more consistent and robust results, the OB population produced no significant results, probably due to their more heterogeneous genome. Thus, standard populations are recommended in studies approaching the long-term effects of pharmacological substance exposure. This study also supports the use of zebrafish as an excellent translational model for evaluating the consequences of early alcohol exposure and searching for treatments. Our results add to the knowledge of the long-term effects of FASD using common zebrafish populations.

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Ethical statement

This research was submitted to the Animal Ethics Committee (CEUA/UFRN) under protocol no. 042/2019 and approved by reference no. 188.042/2019, which deals with the production, maintenance, and or use of animals of the phylum Chordata, and subphylum Vertebrata (except humans). This study is by Law no. 11,794 of October 8, 2008, Decree 6899 of July 15, 2009, and the guidelines established by the National Council for the Control of Animal Experimentation (CONCEA).

CRediT authorship contribution statement

Thaís Agues-Barbosa: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Augusto Monteiro de Souza:** Methodology, Formal analysis, Writing – review & editing. **Jackson Nazareno Gomes de Lima:** Methodology, Formal analysis. **Ana Carolina Luchiari:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

References

Abozaid, A., Trzuskot, L., Najmi, Z., Paul, I., Tsang, B., Gerlai, R., 2020. Developmental stage and genotype dependent behavioral effects of embryonic alcohol exposure in zebrafish larvae. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 97, 109774. https://doi.org/10.1016/j.pnpbp.2019.109774.

Agues-Barbosa, T., da Silva Junior, F.C., Gomes-de-Lima, J.N., Batistuzzo de Medeiros, S. R., Luchiari, A.C., 2022. Behavioral genetics of alcohol's effects in three zebrafish (Danio rerio) populations. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 114, 110495. https://doi.org/10.1016/j.pnpbp.2021.110495.

Alsakran, A., Kudoh, T., 2021. Zebrafish as a model for fetal alcohol spectrum disorders. Front. Pharmacol. 12, 1–7. https://doi.org/10.3389/fphar.2021.721924.

Azimian Zavareh, P., Silva, P., Gimhani, N., Atukorallaya, D., 2022. Effect of embryonic alcohol exposure on craniofacial and skin melanocyte development: insights from zebrafish (Danio rerio. Toxics 10. https://doi.org/10.3390/toxics10090544.

Baggio, S., Mussulini, B.H., de Oliveira, D.L., Gerlai, R., Rico, E.P., 2018. Embryonic alcohol exposure leading to social avoidance and altered anxiety responses in adult zebrafish. Behav. Brain Res. 352, 62–69. https://doi.org/10.1016/j.bbr.2017.08.039.

Baiamonte, M., Parker, M.O., Vinson, G.P., Brennan, C.H., 2016. Sustained effects of developmental exposure to ethanol on Zebrafish anxiety-like behaviour. PLoS One 11, 1–14. https://doi.org/10.1371/journal.pone.0148425.

Bailey, J.M., Oliveri, A.N., Zhang, C., Frazier, J.M., Mackinnon, S., Cole, G.J., Levin, E.D., 2015. Long-term behavioral impairment following acute embryonic ethanol exposure in zebrafish. Neurotoxicol. Teratol. 48, 1–8. https://doi.org/10.1016/j.ntt.2015.01.005.

Baker, M.R., Goodman, A.C., Santo, J.B., Wong, R.Y., 2018. Repeatability and reliability of exploratory behavior in proactive and reactive zebrafish, Danio rerio. Sci. Rep. 8, 1–9. https://doi.org/10.1038/s41598-018-30630-3.

Burton, D.F., Zhang, C., Boa-Amponsem, O., Mackinnon, S., Cole, G.J., 2017. Long-term behavioral change as a result of acute ethanol exposure in zebrafish: Evidence for a role for sonic hedgehog but not retinoic acid signaling. Neurotoxicol. Teratol. 61, 66–73. https://doi.org/10.1016/j.ntt.2017.01.006.

Buske, C., Gerlai, R., 2011. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol. Teratol. 33, 698–707. https://doi.org/10.1016/j.ntt.2011.05.009.

Cadena, P.G., Cadena, M.R.S., Sarmah, S., Marrs, J.A., 2020. Folic acid reduces the ethanol-induced morphological and behavioral defects in embryonic and larval zebrafish (Danio rerio) as a model for fetal alcohol spectrum disorder (FASD. Reprod. Toxicol. 96, 249–257. https://doi.org/10.1016/j.reprotox.2020.07.013.

Cagni, P., Komorowski, M., Melo, G.C., Lima, T., Barros, M., 2012. Diazepam-induced decrease in anxiety-like behaviors of marmoset monkeys exposed to a novel open-field. Pharmacol. Biochem. Behav. 100, 518–521. https://doi.org/10.1016/j.pbb.2011.10.020.

Chung, D.D., Pinson, M.R., Bhenderu, L.S., Lai, M.S., Patel, R.A., Miranda, R.C., 2021. Toxic and teratogenic effects of prenatal alcohol exposure on fetal development, adolescence and adulthood. Int. J. Mol. Sci. 22. https://doi.org/10.3390/ijms22168785.

Cole, G.J., Zhang, C., Ojiaku, P., Bell, V., Devkota, S., Mukhopadhyay, S., 2012. Effects of Ethanol Exposure on Nervous System Development in Zebrafish, International Review of Cell and Molecular Biology. Elsevier. https://doi.org/10.1016/B978-0-12-394310-1.00007-2.

Facciol, A., Gerlai, R., 2020. Zebrafish Shoaling, its behavioral and neurobiological mechanisms, and its alteration by embryonic alcohol exposure: a review. Front. Behav. Neurosci. 14, 1–12. https://doi.org/10.3389/fnbeh.2020.572175.

Fernandes, Y., Gerlai, R., 2009. Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish. Alcohol. Clin. Exp. Res. 33, 601–609. https://doi.org/10.1111/j.1530-0277.2008.00874.x.

Fernandes, Y., Tran, S., Abraham, E., Gerlai, R., 2014. Embryonic alcohol exposure impairs associative learning performance in adult zebrafish. Behav. Brain Res. 265, 181–187. https://doi.org/10.1016/j.bbr.2014.02.035.

Fernandes, Y., Rampersad, M., Gerlai, R., 2015. Impairment of social behaviour persists two years after embryonic alcohol exposure in zebrafish: A model of fetal alcohol spectrum disorders. Behav. Brain Res. 292, 102–108. https://doi.org/10.1016/j.bbr.2015.05.060.

Fernandes, Y., Buckley, D.M., Eberhart, J.K., 2018. Diving into the world of alcohol teratogenesis: a review of zebrafish models of fetal alcohol spectrum disorder. Biochem. Cell Biol. 96, 88–97. https://doi.org/10.1139/bcb-2017-0122.

Gerlai, R., Ahmad, F., Prajapati, S., 2008. Differences in acute alcohol-induced behavioral responses among zebrafish populations. Alcohol. Clin. Exp. Res. 32, 1763–1773. https://doi.org/10.1111/j.1530-0277.2008.00761.x.

de Graaf-Peters, V.B., Hadders-Algra, M., 2006. Ontogeny of the human central nervous system: what is happening when. Early Hum. Dev. 82, 257–266. https://doi.org/10.1016/j.earlhumdev.2005.10.013.

Harvey, R.E., Berkowitz, L.E., Hamilton, D.A., Clark, B.J., 2019. The effects of developmental alcohol exposure on the neurobiology of spatial processing. Neurosci. Biobehav. Rev. 107, 775–794. https://doi.org/10.1016/j.neubiorev.2019.09.018.

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., Weng, W., Bally-Cuif, L., Schneider, H., 2013. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10, 70–86. https://doi.org/10.1089/zeb.2012.0861.

Lange, M., Neuzeret, F., Fabreges, B., Froc, C., Bedu, S., Bally-Cuif, L., Norton, W.H.J., 2013. Inter-individual and inter-strain variations in zebrafish locomotor ontogeny. PLoS One 8. https://doi.org/10.1371/journal.pone.0070172.

Lange, S., Probst, C., Gmel, G., Rehm, J., Burd, L., Popova, S., 2017. Global prevalence of fetal alcohol spectrum disorder among children and youth. JAMA Pediatr. 171, 948. https://doi.org/10.1001/jamapediatrics.2017.1919.

Lange, S., Shield, K., Rehm, J., Anagnostou, E., Popova, S., 2019. Fetal alcohol spectrum disorder: neurodevelopmentally and behaviorally indistinguishable from other neurodevelopmental disorders. BMC Psychiatry 19, 1–10. https://doi.org/10.1186/s12888-019-2289-y.

La-Vu, M., Tobias, B.C., Schuette, P.J., Adhikari, A., 2020. To approach or avoid: an introductory overview of the study of anxiety using rodent assays. Front. Behav. Neurosci. 14, 1–7. https://doi.org/10.3389/fnbeh.2020.00145.

Leite-Ferreira, M.E., Araujo-Silva, H., Luchiari, A.C., 2019. Individual differences in hatching time predict alcohol response in zebrafish. Front. Behav. Neurosci. 13, 1–9. https://doi.org/10.3389/fnbeh.2019.00166.

Little, B., Sud, N., Nobile, Z., Bhattacharya, D., 2021. Teratogenic effects of maternal drug abuse on developing brain and underlying neurotransmitter mechanisms. Neurotoxicology 86, 172–179. https://doi.org/10.1016/j.neuro.2021.08.007.

Loucks, E., Carvan, M.J., 2004. Strain-dependent effects of developmental ethanol exposure in zebrafish. Neurotoxicol. Teratol. 26, 745–755. https://doi.org/10.1016/j.ntt.2004.06.017.

Mahabir, S., Chatterjee, D., Gerlai, R., 2014. Strain dependent neurochemical changes induced by embryonic alcohol exposure in zebrafish. Neurotoxicol. Teratol. 41, 1–7. https://doi.org/10.1016/j.ntt.2013.11.001.

Mattson, S.N., Bernes, G.A., Doyle, L.R., 2019. Fetal alcohol spectrum disorders: a review of the neurobehavioral deficits associated with prenatal alcohol exposure. Alcohol. Clin. Exp. Res 43, acer.14040. https://doi.org/10.1111/acer.14040.

Maximino, C., de Brito, T.M., da Silva Batista, A.W., Herculano, A.M., Morato, S., Gouveia, A., 2010. Measuring anxiety in zebrafish: a critical review. Behav. Brain Res. 214, 157–171. https://doi.org/10.1016/j.bbr.2010.05.031.

May, P.A., Baete, A., Russo, J., Elliott, A.J., Blankenship, J., Kalberg, W.O., Buckley, D., Brooks, M., Hasken, J., Abdul-Rahman, O., Adam, M.P., Robinson, L.K., Manning, M., Hoyme, H.E., 2014. Prevalence and characteristics of fetal alcohol spectrum disorders. Pediatrics 134, 855–866. https://doi.org/10.1542/peds.2013-3319.

Mayfield, R.D., Harris, R.A., Schuckit, M.A., 2008. Genetic factors influencing alcohol dependence. Br. J. Pharmacol. 154, 275–287. https://doi.org/10.1038/bjp.2008.88.

Pan, Y., Chatterjee, D., Gerlai, R., 2012. Strain dependent gene expression and neurochemical levels in the brain of zebrafish: focus on a few alcohol related targets. Physiol. Behav. 107, 773–780. https://doi.org/10.1016/j.physbeh.2012.01.017.

Pannia, E., Tran, S., Rampersad, M., Gerlai, R., 2014. Acute ethanol exposure induces behavioural differences in two zebrafish (Danio rerio) strains: a time course analysis. Behav. Brain Res. 259, 174–185. https://doi.org/10.1016/j.bbr.2013.11.006.

Parker, M.O., Annan, L.V., Kanellopoulos, A.H., Brock, A.J., Combe, F.J., Baiamonte, M., Teh, M.T., Brennan, C.H., 2014. The utility of zebrafish to study the mechanisms by which ethanol affects social behavior and anxiety during early brain development. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 55, 94–100. https://doi.org/10.1016/j.pnpbp.2014.03.011.

Paul, I., Tsang, B., Gerlai, R., 2020. Short exposure to moderate concentration of alcohol during embryonic development does not alter gross morphology in Zebrafish. Zebrafish 17, 253–260. https://doi.org/10.1089/zeb.2020.1872.

Petrenko, C.L.M., Tahir, N., Mahoney, E.C., Chin, N.P., 2014. Prevention of secondary conditions in fetal alcohol spectrum disorders: Identification of systems-level barriers. Matern. Child Health J. 18, 1496–1505. https://doi.org/10.1007/s10995-013-1390-y.

Pinheiro-da-Silva, J., Araujo-Silva, H., Luchiari, A.C., 2021. Does early ethanol exposure increase seeking-like behavior in zebrafish? Int. J. Dev. Neurosci. 81, 416–427. https://doi.org/10.1002/jdn.10112.

Pinheiro-Da-Silva, J., Agues-Barbosa, T., Carolina Luchiari, A., 2020. Embryonic exposure to ethanol increases anxiety-like behavior in fry zebrafish. Alcohol Alcohol. 55, 581–590. https://doi.org/10.1093/ALCALC/AGAA087.

Popova, S., Dozet, D., Akhand Laboni, S., Brower, K., Temple, V., 2022. Why do women consume alcohol during pregnancy or while breastfeeding. Drug Alcohol Rev. 41, 759–777. https://doi.org/10.1111/dar.13425.

Ramlan, N.F., Sata, N.S.A.M., Hassan, S.N., Bakar, N.A., Ahmad, S., Zulkifli, S.Z., Abdullah, C.A.C., Ibrahim, W.N.W., 2017. Time dependent effect of chronic embryonic exposure to ethanol on zebrafish: morphology, biochemical and anxiety alterations. Behav. Brain Res. 332, 40–49. https://doi.org/10.1016/j.bbr.2017.05.048.

Rice, D., Barone, S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108, 511–533. https://doi.org/10.1289/ehp.00108s3511.

Rico, E.P., Rosemberg, D.B., Seibt, K.J., Capiotti, K.M., Da Silva, R.S., Bonan, C.D., 2011. Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol. Teratol. 33, 608–617. https://doi.org/10.1016/j.ntt.2011.07.007.

Seguin, D., Gerlai, R., 2018. Fetal alcohol spectrum disorders: Zebrafish in the analysis of the milder and more prevalent form of the disease. Behav. Brain Res. 352, 125–132. https://doi.org/10.1016/j.bbr.2017.10.005.

Seibenhener, M.L., Wooten, M.C., 2015. Use of the open field maze to measure locomotor and anxiety-like behavior in mice. J. Vis. Exp. 1–6. https://doi.org/10.3791/52434.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol. Rev. 83, 18–34. https://doi.org/10.1111/j.1469-185x.2007.00030.x.

Suresh, S., Abozaid, A., Tsang, B., Gerlai, R., 2021. Exposure of parents to alcohol alters behavior of offspring in zebrafish. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 111, 110143. https://doi.org/10.1016/j.pnpbp.2020.110143.

Trevarrow, B., Robison, B., 2004. Genetic backgrounds, standard lines, and husbandry of zebrafish. Methods Cell Biol. 2004, 599–616. https://doi.org/10.1016/s0091-679x(04)77032-6.

Tsang, B., Ansari, R., Gerlai, R., 2019. Dose dependent behavioral effects of acute alcohol administration in zebrafish fry. Pharmacol. Biochem. Behav. 179, 124–133. https://doi.org/10.1016/j.pbb.2019.02.011.

Varga, Z.K., Zsigmond, Á., Pejtsik, D., Varga, M., Demeter, K., Mikics, É., Haller, J., Aliczki, M., 2018. The swimming plus-maze test: a novel high-throughput model for assessment of anxiety-related behaviour in larval and juvenile zebrafish (Danio rerio. Sci. Rep. 8, 1–11. https://doi.org/10.1038/s41598-018-34989-1.

Wall, T.L., Luczak, S.E., Hiller-Sturmhöfel, S., 2016. Biology, genetics, and environment: underlying factors influencing alcohol metabolism. Alcohol Res. 38, 59–68.

Yoshida, M., 2022. Incorporating ventilatory activity into a novel tank test for evaluating drug effects on zebrafish. Physiol. Behav. 257, 113978 https://doi.org/10.1016/j.physbeh.2022.113978.