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Does early ethanol exposure increase seeking-like behavior in zebrafish?

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Abstract

Fetal alcohol spectrum disorder (FASD) is the most common cause of birth defects. The severe variations are in fetal alcohol syndrome (FAS) but the most frequent cases are alcohol-related neurodevelopmental disorder (ARND), which is of a difficult diagnosis. ARND characteristics include impaired social behavior, anxiety and depression prevalence, cognitive deficits, and an increased chance for drug addiction. Here, we aimed to test whether early alcohol exposure leads to later alcohol preference. We hypothesize that early alcohol exposure increases the reinforcing effects on later experiences, raising the chance of addiction in adult life. Lately, the zebrafish has been a valuable model on alcohol research, allowing embryonic exposure and the study of the ontogenetic effects. For this, embryos were exposed to three different alcohol treatments: 0.0%, 0.25% and 0.5%, for 2 hr, at 24-hr post-fertilization. Then we evaluated the effects of embryonic alcohol exposure on conditioned place preference in two developmental stage: fry (10 days post-fertilization (dpf)) and young (90 dpf) zebrafish. Results show that control fish presented alcohol associative learning, which means, changes in place preference due to alcohol exposure, at both ontogenetic phases. However, zebrafish exposed to 0.25 and 0.5% alcohol during embryogenesis did not show conditioning response at any evaluated stage. These results suggest perception and cognitive deficits due to embryonic alcohol exposure that can alter alcohol responsiveness throughout a lifetime. Although low alcohol doses do not provoke malformation, it has been shown to induce several neurological and behavioral changes that are termed as Alcohol-Related Neurodevelopmental Disorders. These results may contribute to future investigations on how embryonic exposure affects the neurocircuitry related to perception and associative learning processing.

KEYWORDS

alcohol, ARND, seeking-like behavior, zebrafish

Abbreviations: ANOVA, analysis of variance; ARND, alcohol-related neurodevelopmental disorder; CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; CEUA, Committee for Animal Use of Federal University of Rio Grande do Norte; CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico; CPP, conditioned place preference; dpf, days post-fertilization; EtOH, ethanol; FAS, fetal alcohol syndrome; FASD, fetal alcohol spectrum disorder; hpf, hours post-fertilization.

1 | INTRODUCTION

Alcohol (ethanol—EtOH) is a psychotropic substance and a legal drug of great social acceptance, known as the most used mood-altering drug worldwide (Kanny et al., 2013; Schweinsburg et al., 2011; Siqueira et al., 2007). Because the alcohol business is one of the most profitable segments of the economy, its consumption is often encouraged, although the consequences from alcoholism are among the most costly and devastating for health and society (Harwood et al., 1998; Lima, 2003; OMS, 2002).

Alcohol indiscriminate consumption by pregnant women has been a growing social problem (Jacobs et al., 2000; Tebeka et al., 2020). Gestational alcohol exposure has often been associated with teratogen effects, developmental delays, and infant mortality (Barr et al., 2003; Bilotta et al., 2004; May et al., 2009; Ornoy & Ergaz, 2010). Fetal alcohol syndrome (FAS) is a tough clinical condition that causes severe changes in fetal development and presents marked signals afterbirth, as growth deficit, mental problems, and facial changes characterized by microphthalmia, retrognathism, and absence of nasolabial sulcus (Lima, 2003; Lutte et al., 2018; Morse, 1998). Currently, the term fetal alcohol spectrum disorders (FASD) is used to define the full range of alcohol-induced birth defects, which vary from a very severe state as the syndrome to milder cases of alcohol-related neurodevelopmental disorder (ARND) (Arenzana et al., 2006; Gil-Mohapel et al., 2019). However, the biggest concern is still the manifestation of long-term cognitive and behavioral deficits that are not associated with morphological alterations and appear under conditions of moderate consumption of alcohol during pregnancy that leads to child learning difficulties and behavioral issues (Eckardt et al., 1998; Guerri et al., 2009).

It is well-known that alcohol abuse seems to involve genetic predisposition, for example, twins have an estimated heritability of 50%–60% for genetic risk components of alcoholism (Heath et al., 1997; Malone et al., 2014), and siblings and first-degree relatives on individuals with alcohol abuse history are three to five times more likely to develop an addiction (Cotton, 1979). However, studies suggest that gene expression is determined not only by the DNA code but from a set of epigenetic effects, which are modulated by the environment/experiences (Allis et al., 2007; Berger et al., 2009; Horwitz et al., 2003; Weaver et al., 2004). Disorders of the cellular epigenetic pattern, such as alcohol metabolism, may result in loss of the tissue identity or abnormal activation of signaling pathways, which lead to neurological problems (Slomko et al., 2012). Therefore, alcohol exposure may trigger many physiological and behavioral changes, and may also be related to the individual predisposition to alcohol search and addiction development (Baker et al., 2018; Mathur et al., 2011).

Although there is substantial literature on the effects of gestational alcohol consumption on developing offspring, there is still a major difficulty in studying the drug effects during initial development in mammals. Fortunately, the zebrafish *(Danio rerio)* is an animal model that presents external fertilization and has gained much popularity in this research area. From the ethics perspective, zebrafish is a good alternative for reduction and refinement to mammalian laboratory species. Still a vertebrate, but more complex and evolutionary closer to humans than other frequently employed non-vertebrate model organisms in medical research areas, such as nematodes or drosophila (Shams et al., 2017). Furthermore, the zebrafish presents more advantages than just its genetic similarity with humans (70%–80% of homology): small size, high fertilization rate, short development time, and embryo development outside the uterus are features that give advantage to the zebrafish research (Gerlai, 2014; Grunwald & Eisen, 2002; Miklósi & Andrew, 2006). Besides, alcohol easily crosses the chorion, facilitating further studies on zebrafish development (Blader & Strähle, 1998; Tran & Gerlai, 2014).

Although alcohol is a central nervous system depressant, it is the initially stimulating action that places this substance at the top of the popularly consumed drugs. The ability to reinforce behaviors is one of the most important characteristics of drugs of abuse, and previous studies show that zebrafish presents behavioral alteration for reinforcing stimuli (Bilotta et al., 2005; Homberg et al., 2004; Mathur & Guo, 2011). Based on that and compiled to zebrafish popularity in behavioral pharmacology, we aimed to test whether early alcohol exposure leads to later alcohol preference. We hypothesize that early alcohol exposure increases the reinforcing effects on later experiences, raising the chance of drug addiction in adult life.

2 | MATERIALS AND METHODS

2.1 | Animals and housing

To obtain the fish used in the following protocol, adult zebrafish (wild type, both sexes, ~6 months old, 1.8 ± 0.44 g) were held at Fish Vivarium (Physiology and Behavior Department—UFRN) and maintained in groups in a recirculating water system with mechanical, biological, and activated carbon filter and a UV light sterilizing unit. Photoperiod was standard at 12-hr light/12-hr dark cycling and temperature (28 ± 1°C), pH (6.8–7.2), and oxygen levels (~6 mg/L) were measured regularly. All protocols were reviewed and approved by the Committee for Animal Use of Federal University of Rio Grande do Norte (CEUA 004002/2017).

To address the main question of this study we analyzed the alcohol effects during embryonic development in two

different phases, fry (10 days post-fertilization (dpf)) and young adults (90 dpf). For this, fish were set up for breeding (2 females: 1 male) during the late afternoon, eggs were collected at the first hour of the light cycle on the following day, and embryos were raised under standard conditions (Westerfield, 2007).

Alcohol exposure occurred always at 24 hr post-fertilization (hpf). This period was chosen based on previously published data showing equivalence to the first trimester of the human pregnancy and the period when fish starts to produce synapses (Carvan et al., 2004; Shan et al., 2015). Zebrafish embryos were divided into three different alcohol treatments: 0.0%, 0.25%, and 0.5%, embryos were randomly allocated to treatment from different breedings essays. Eggs were immersed in their respective concentration for 2 hr and then washed in system water right after. Eggs were maintained in 3-L tanks with clean water and oxygen source. Food was introduced by the 5th dpf with a mixture of live brine shrimp nauplii *(artemia salina)* and very fine powder food (Alcon Alevinos, Alcon, Brazil). Fry fish were tested in a Conditioned Place Preference (CPP) test at 10 dpf. A different set of animals were let to grow up to 90 days. These fish were transferred from the 3-L tanks to 8-L tanks in a recirculating closed system and fed with flake food (Alcon Basic H, Alcon, Brazil) and brine shrimp from the 30th dpf onwards. Adult fish were tested in a Conditioned Place Preference (CPP) test at 90 dpf.

2.2 | Conditioned Place Preference Test (CPP)

In the CPP protocol, a drug and a particular context are paired, and the preference for that is then measured. The conditioned stimulus (CS) is the apparatus itself, made with visual cues such as a pattern on the floor and walls. The unconditioned stimulus (US) is alcohol, and the unconditioned response is the alcohol effect, usually rewarding. The methodology applied here was based on other classical protocols of conditioned place preference commonly tested in zebrafish (Avdesh et al., 2010; Chacon & Luchiari, 2014; Mathur et al., 2011; Ninkovic & Bally-Cuif, 2006; Parker et al., 2016), with slight adaptations between fry and adults, to adjust the apparatus space to animal size.

The testing apparatus consisted of an arena divided in half by distinct visual cues: one striped side in black and white, and the other side completely white. The difference is that to test CPP in fry, Petri dishes were used (6 cm diameter) and for adults, we used 15L tanks (40 cm × 20 cm × 20 cm) (see Figure 1 for details). Conditioning was performed in three phases: *Day 1*, basal preference analysis; *Day 2*, alcohol administration on the "non-preferred" side; *Day 3*, post-drug preference analysis. During the CPP, when the unconditioned stimulus was applied, animals always received Eth 0.5%. This dose has previously been used and shown to cause changes in preference (Lockwood et al., 2004; Parker et al., 2016; Tran

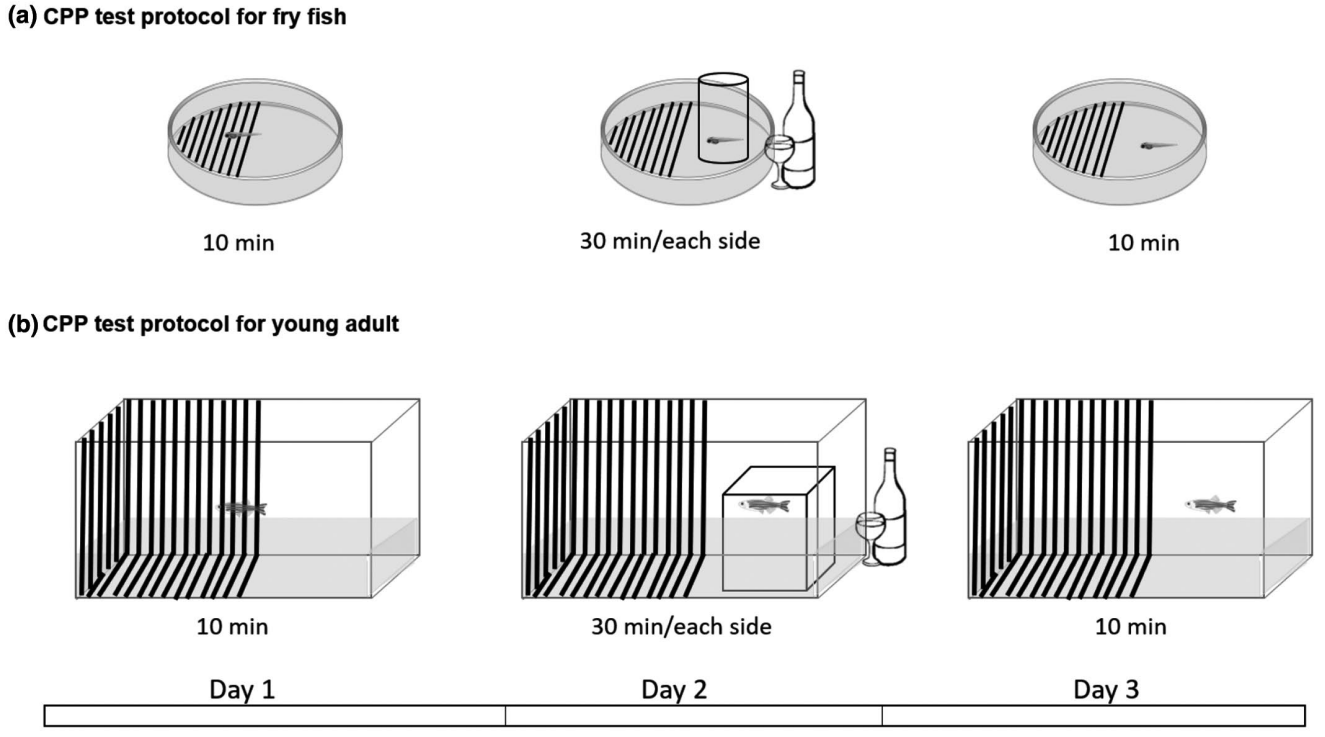

FIGURE 1 Experimental design for Conditioned Place Preference test. Protocol applied to (a) Fry fish (10 dpf) and (b) Adult fish (90 dpf). On day 1 (basal preference) and day 3 (post-treatment preference) fish behavior was recorded for 10 min. On day 2 fish were exposed to 0.0% or 0.5% alcohol at the non-preferred side of the previous day, which means the place fish spent less than 60% of the total time

& Gerlai, 2013). For both protocols, the apparatus water was changed every 3 trials, to reduce any hormonal release by tested fish, confusing the task.

Day 1: Using a fishnet, each animal was gently placed on the white side of the testing tank. Each animal had 15 min to freely explore the arena and behavior was recorded on video for further analysis. The first 5 min in the tank was considered as habituation, while the remaining 10 min was considered for analysis in a tracking software. Location preference was considered when the animal passed 60% or more on one side of the arena. Animals that showed no preference for the location were discarded from the next phases.

Day 2: After analyzing individual preference, animals were placed back in the arena, but this time they were placed inside a smaller tank (with transparent sides and bottoms so they could recognize the visual cues of the outside area) for alcohol exposure. Each animal remained 30 min at the non-preferred side, with 0.5% alcohol added in the smaller tank, followed by 30 min on the previously preferred side with only water in the tank. A control group was exposed to 0.0% alcohol (clean water) on both sides of the arena, 30 min on each side.

Day 3: The same Day 1 protocol was applied to check for changes in place preference after drug exposure.

2.3 | Experimental treatments

All eggs used in this study were exposed to 0.0%, 0.25%, or 0.5% alcohol at 24 hpf, animals were randomly assigned to each treatment conditions, and groups were blinded during all experimental phase. Animals were let grow and tested for CPP at 10 dpf and 90 dpf, when fish were exposed to 0.0% (control) or 0.5% alcohol during the conditioning phase (day 2 described above). Fish tested at 10 dpf were not the same animals tested at 90 dpf. This experimental design produced six alcohol treatment groups for each ontogenetic stage, $n = 10$ animals/group. Final sample size was determined by a initial pilot study followed by a power calculation (G * Power 3.1.9.2), computing the required total sample size for an effect size of 0.25, α error prop 0.05, and actual power of 0.97. We refer to according to the concentration used for embryo (E) and concentration used for the CPP test (F for fry and A for Adult) as follows: E0.0F0.0, E0.0F0.5, E0.25F0.0, E0.25F0.5, E0.5F0.0 and E0.5F0.5 at the fry stage and E0.0A0.0, E0.0A0.5, E0.25A0.0, E0.25A0.5, E0.5A0.0 and E0.5A0.5 at the young adult stage.

2.4 | Data analysis

All tests were video recorded, and behavior was analyzed in tracking software (ZebTrack (Pinheiro-da-Silva et al., 2017), developed in MATLAB platform—R2014a, Math Works, Natick, MA). Time spent in each area of the CPP tank and locomotor parameters of swimming speed, distance traveled, and immobility were evaluated. Data were previously tested for normality using the Shapiro-Wilk test and Levene's test to verify data homoscedasticity. Next, we accessed a One-Way Analysis of Variance (ANOVA) for normal data or the Kruskal Wallis test for non-normal data (Tukey HSD post hoc test followed both of the choices) to test intergroup differences in average speed, maximum speed, immobility time, and total distance traveled during the test day. A comparison between experimental groups and time on the non-preferred area (before vs. after alcohol exposure) was accessed by Two-Way RM ANOVA considering as factors treatment (alcohol 0.0 or 0.5%) and day of testing (day 1-basal preference or day 3-post alcohol preference), followed by the Tukey post hoc test. R Studio software (R Core Team, 2019) was used to perform statistical analyses. The significance level was established at $p < .05$.

3 | RESULTS

3.1 | Fry fish

Embryos exposed to 0.0%, 0.25%, or 0.5% alcohol were tested at the fry stage of 10 dpf in a CPP protocol using 0.0% alcohol (control) or 0.5% alcohol. Basal preference was observed on day 1 and preference changes due to alcohol exposure were checked on day 3. Results as shown in Figure 2.

For E0.0 tested at the fry stage (Figure 2a), Two-Way RM ANOVA revealed non-significant effect of treatment ($F_{(1,18)} = 2.20$; $p = .15$), but statistically significant effect of day ($F_{(1,18)} = 8.92$; $p = .008$). The interaction terms treatment versus day was found non-significant ($F_{(1,18)} = 2.52$; $p = .13$). For E0.25 tested at the fry stage (Figure 2b), Two-Way RM ANOVA showed statistically significant effect of treatment ($F_{(1,18)} = 9.20$; $p = .005$) and day ($F_{(1,18)} = 11.34$; $p = .004$). The interaction terms treatment versus day was shown significant ($F_{(1,18)} = 8.52$; $p = .007$). For E0.5 tested at the fry stage (Figure 2c), Two-Way RM ANOVA indicated non- significant effect of treatment ($F_{(1,18)} = 3.12$; $p = .09$), day ($F_{(1,18)} = 3.72$; $p = .07$), and interaction terms treatment versus day ($F_{(1,18)} = 0.01$; $p = .90$).

Locomotor parameters are depicted in Figure 3. One-Way ANOVA showed no statistical significance for average speed ($F_{(5,48)} = 1.768$; $p = .137$; Figure 3a), maximum speed ($F_{(5,48)} = 1.602$; $p = .178$; Figure 3b) and total distance traveled ($F_{(5,48)} = 1.89$; $p = .114$; Figure 3c). For immobility time, comparation made by Kruskal-Wallis test showed statistical significance between treatments ($\chi^2 = 22.819$; $df = 5$; $p < .001$; Figure 3d). Tukey HSD test showed that E0.0F0.0 presented lower immobility time than E0.0F0.5, E0.25F0.0 and E0.25F0.5 groups.

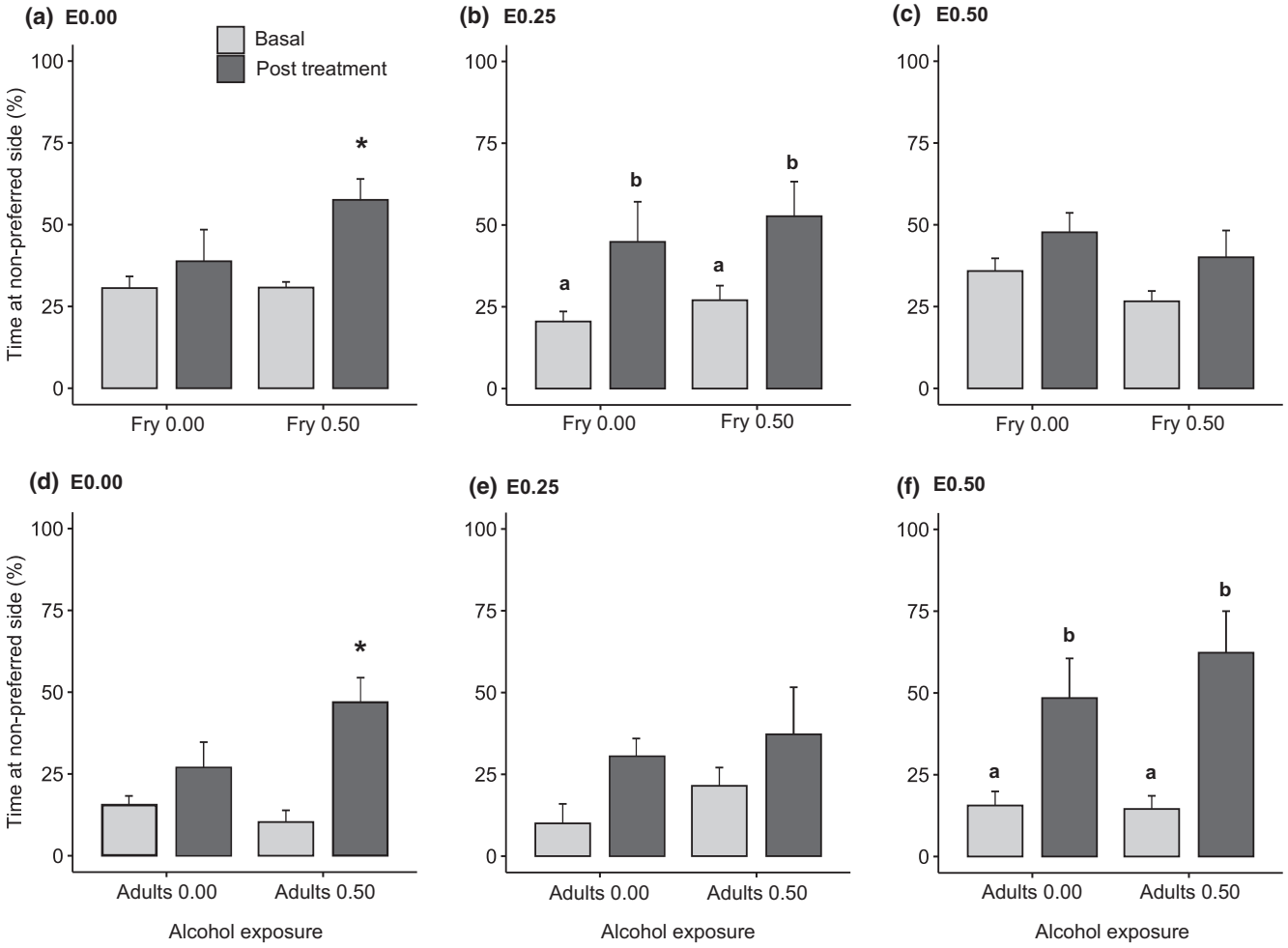

FIGURE 2 Conditioned Place Preference (CPP) behavior in fry and adult zebrafish. Fish were embryonically exposed to 0.0% (E0.0), 0.25% alcohol (E0.25) or 0.5% alcohol (E0.5) from 24 to 26 hpf. At 10 dpf, fry fish were tested for CPP (a, b, and c) when they were exposed to 0.0 or 0.5% alcohol at the non-preferred side of the arena. At 90 dpf, adult fish were tested for CPP (d, e, and f) when they were exposed to 0.0 or 0.5% alcohol at the non-preferred side of the arena. Bars represent average (± *SEM*) time spent at the non-preferred side of the arena, defined as the area where fish spent less than 60% of the total time (10 min). On day 1 (basal preference) and day 3 (post-treatment preference) fish behavior was recorded for 10 min. (*) and different letters indicate statistical significance (Two-Way ANOVA; $p < .05$)

3.2 | Adult fish

Embryos exposed to 0.0%, 0.25% or 0.5% alcohol were tested at the young adult stage of 90 dpf in a CPP protocol using 0.0% alcohol (control) or 0.5% alcohol. Preference is shown in Figure 2.

For those fish treated with 0.0% alcohol during embryogenesis (E0.0) and tested at the young adult stage (Figure 2d), Two-Way RM ANOVA revealed non-significant effect of treatment ($F_{(1,18)} = 0.00$; $p = .99$), but statistically significant effect of day ($F_{(1,18)} = 5.54$; $p = .03$). The interaction terms treatment versus day was found non-significant ($F_{(1,18)} = 0.43$; $p = .51$). For E0.25 tested at the young adult stage (Figure 2e), Two-Way RM ANOVA showed non-significant effect of treatment ($F_{(1,18)} = 0.22$; $p = .64$) and statistically significant effect of day ($F_{(1,18)} = 5.11$; $p = .04$). The interaction terms treatment versus day was shown non-significant ($F_{(1,18)} = 0.44$; $p = .52$). For E0.5 tested at the young adult stage (Figure 2f), Two-Way RM ANOVA indicated non-significant effect of treatment ($F_{(1,18)} = 0.53$; $p = .47$), but significant effect of day ($F_{(1,18)} = 17.13$; $p < .01$). Interaction terms treatment versus day was shown non-significant ($F_{(1,18)} = 0.58$; $p = .45$).

For locomotor parameters obtained from young zebrafish, One-Way ANOVA showed no statistical significance for average speed ($F_{(5,45)} = 1.03$; $p = .41$; Figure 4a), maximum speed ($F_{(5,45)} = 0.68$; $p = .63$; Figure 4b) and total distance traveled ($F_{(5,45)} = 1.46$; $p = .22$; Figure 4c). For immobility time, comparation made by Kruskal-Wallis test showed statistical significance ($H = 11.1$, $df = 5$, $p = .04$; Figure 4d) between the treatments. Tukey HSD test indicated that E0.50A0.5 presented higher immobility time than E0.0A0.0.

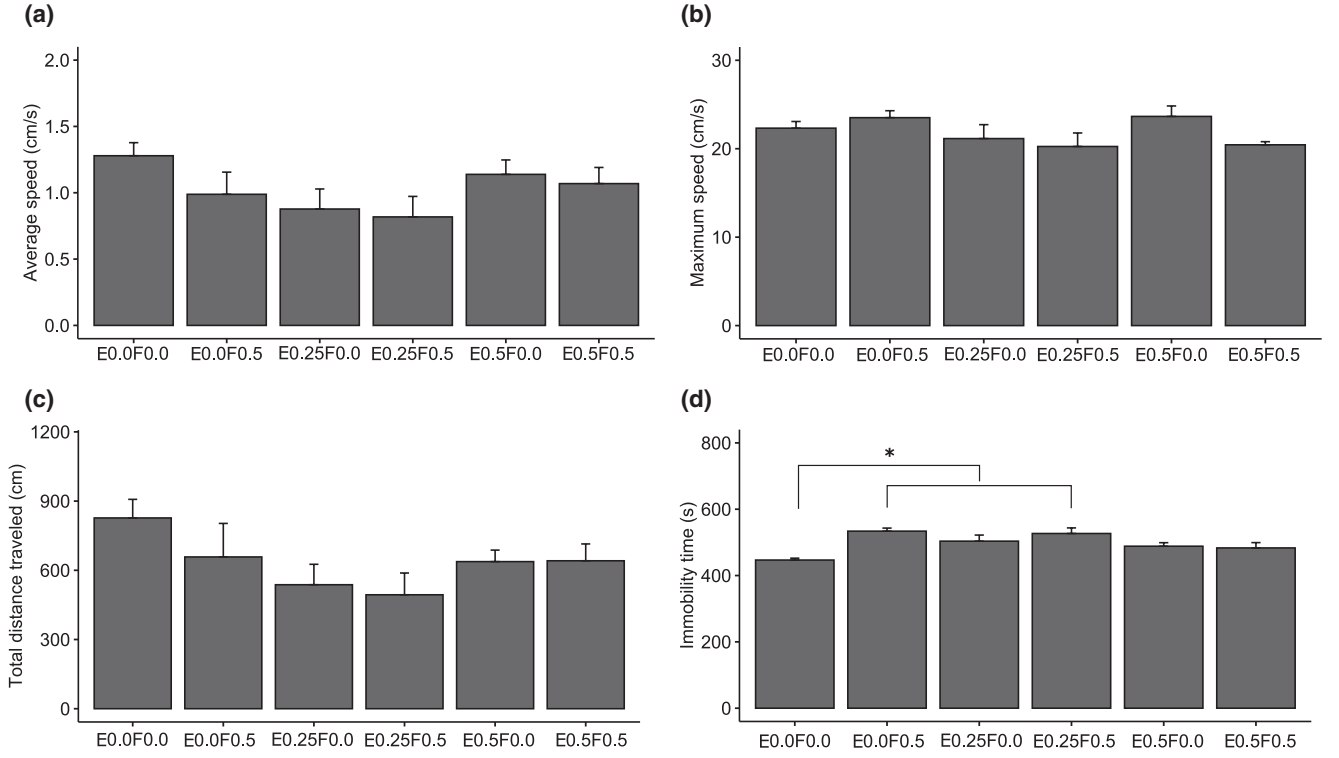

FIGURE 3 Locomotor parameters were obtained from fry fish (10 dpf) on day 3 of the CPP test. Group nomenclature is based on embryonic exposure to alcohol (E0.0, E0.25, or E0.5) and concentration used for the CPP test (F for fry: F0.0 or F0.5). (A) average swimming speed, (B) maximum swimming speed, (C) total distance traveled, and (D) time immobile. Bars represent average ± *SEM*. * indicates statistical significance between groups (Kruskal-Wallis test, $p < .05$)

4 | DISCUSSION

The embryonic exposure to high alcohol doses has been shown to cause teratogenic effects (Arenzana et al., 2006; Becker et al., 1996) included in the Fetal Alcohol Syndrome (FAS) cases. Although low alcohol doses do not provoke malformation, it has been previously shown to induce several neurological and behavioral changes that are termed as Alcohol-Related Neurodevelopmental Disorders (ARND) (Hoyme et al., 2005). This milder form of the disease is much more frequent, and research on diagnosis and treatment is needed. It has recently been shown that ARND causes anxiety-like behavior that can be observed at early developmental stages in zebrafish (Pinheiro-da-Silva et al., 2020). Other abnormal behaviors that are observed at the adult stage include impaired social behavior (Buske & Gerlai, 2011; Carvan et al., 2004; Fernandes & Gerlai, 2009), depressive-related behaviors (Burton et al., 2017; Parker et al., 2014), and cognitive deficits (Carvan et al., 2004). In the current study, alcohol conditioned place preference (changes in place preference due to alcohol exposure) following embryonic alcohol exposure has been evaluated in fry (10 dpf) and young adult (90 dpf) zebrafish. Although control fish showed alcohol associative learning at both ontogenetic phases, zebrafish exposed to 0.25% and 0.5% alcohol during embryogenesis did not show conditioning response at the fry (Figure 2b and c) or the young adult stage (Figure 2e and f). These results suggest perception and/or cognitive deficits due to embryonic alcohol exposure that can alter alcohol responsiveness later on.

Zebrafish embryos that were not exposed to alcohol (E0.0) presented place preference change after alcohol exposure both at fry (Figure 2a) and adult (Figure 2d) stages of development. These findings confirm previous studies in which adult zebrafish showed associative learning in conditioned place preference (CPP) protocols using alcohol (Chacon & Luchiari, 2014; Kily et al., 2008; Mathur et al., 2011; Parmar et al., 2011), and other addictive drugs such as cocaine, amphetamines, morphine, and nicotine (Darland & Dowling, 2001; Lau et al., 2006; Ninkovic & Bally-Cuif, 2006; Parmar et al., 2011). Parker and colleagues (2016) also tested alcohol's effects during brain ontogeny and saw an enhanced preference for the drug-conditioned place. In addition, the authors observed changes in specific gene expression. In these studies, the reinforcing effects of the drugs are confirmed after a single exposure. The conditioned response appears after the drug exposure is paired with a specific place because the brain's reward areas are connected to the limbic system centers for motivation and memory (McLellan et al., 2000). In this sense, the exposure to the same place/environment recreates the memory of the drug experience and triggers a search

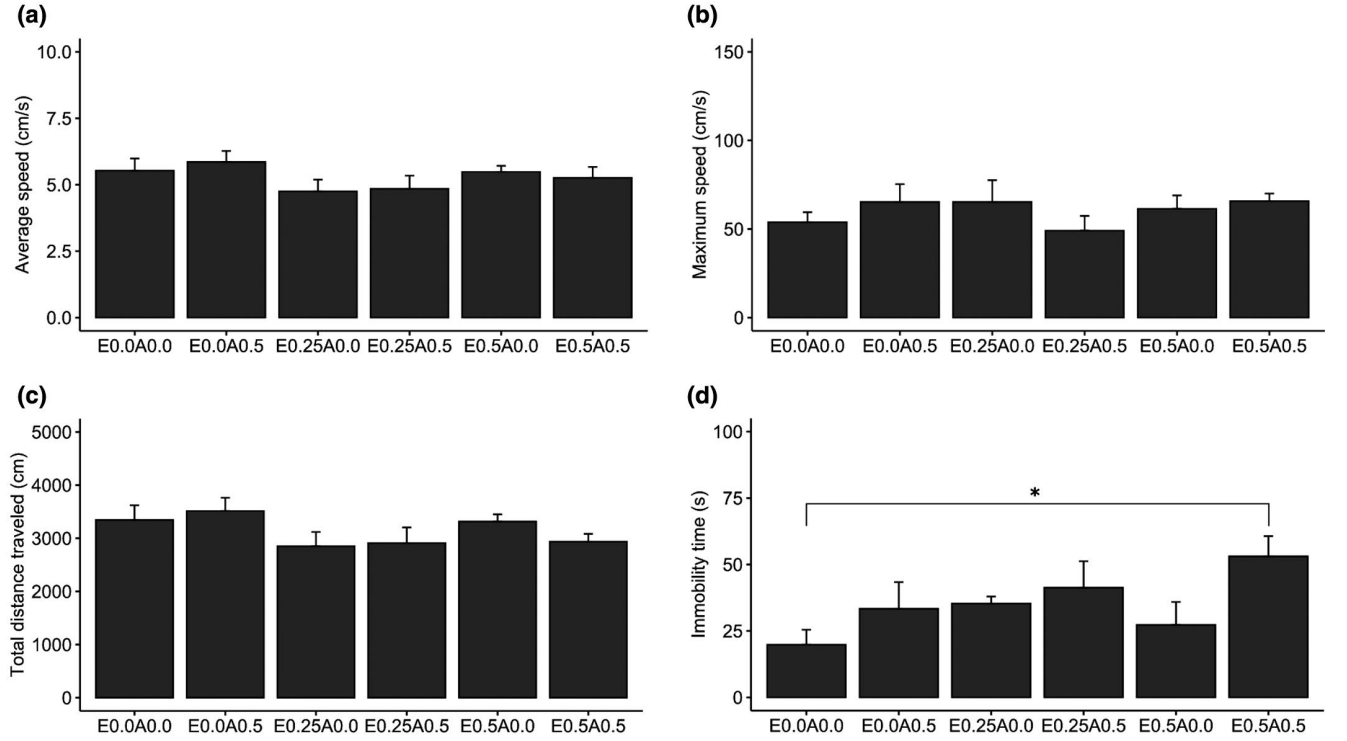

FIGURE 4 Locomotor parameters were obtained from adult fish (90 dpf) on day 3 of the CPP test. Group nomenclature is based on embryonic exposure to alcohol (E0.0, E0.25, or E0.5) and concentration used for the CPP test (A for adult: A0.0 or A0.5). (A) average swimming speed, (B) maximum swimming speed, (C) total distance traveled, and (D) time immobile. Bars represent average ± *SEM*. * indicates statistical significance between groups (Kruskal-Wallis test, $p < .05$)

for the same sensation. In the present study, we have shown that a single exposure to alcohol at 10 dpf (fry stage) led to seeking behavior similar to adult fish.

The zebrafish is a translational model that allows controlled early alcohol exposure to test-seeking behavior at different developmental stages. In this sense, we aimed at testing whether embryonic alcohol exposure could be considered a triggering factor for craving in the present study. It is known that environmental factors influence the vulnerability to alcohol use and abuse (Collier, 2018; Guerri & Pascual, 2010; Rose et al., 2004), however, the adult brain is already fully developed while during embryogenesis different brain areas develop at different times and velocity (Andersen et al., 2008; Pechtel et al., 2014). Thus, early alcohol exposure may have more jeopardizing effects for the individual's life than the alcohol effects in the mature brain (adult response). Despite the CPP task being very popular in adult zebrafish, little is known about this behavior's genesis concerning alcohol exposure time. Here, fish exposed to alcohol during embryogenesis were tested for CPP at 10 dpf (fry) and 90 dpf (young adults).

Although the CCP was observed in control fry (E0.0) after 0.5% alcohol exposure, embryonic alcohol-exposed zebrafish (E0.25 and E0.5) did not show associative response in fry or adult ages (Figure 2). These animals showed preference consistency or changed preference independent of alcohol-place association. Previous studies using a similar design to ours for embryonic alcohol exposure found several behavioral differences in zebrafish larvae and older fish (Baiamonte et al., 2016; Bailey et al., 2015; Parker et al., 2014). In contrast, we found no further alcohol associative learning in zebrafish exposed to 0.25% or 0.5% alcohol. The neurocircuitry underlying CPP behavior in fish has been characterized (O'Connell & Hofmann, 2011), but the effects of embryonic alcohol exposure to this response are unknown. It is possible that embryonic ethanol exposure disrupts associative learning (Fernandes et al., 2014) and affects associative response regardless of the unconditioned stimulus (Parmar et al., 2011). Cleal and Parker (2018) have already discussed that ethanol exposure during development could relate to subtle effects on decision-making processes. Although the consistency of the reaction and other associative learning tests would be required to test this hypothesis properly. Moreover, it will be of great interest to characterize the zebrafish reward circuitry and the effects of early ethanol exposure on it. For instance, how the dopaminergic system is affected by early ethanol exposure is not known.

Our study was based on the assumption that Alcohol-Related Neurodevelopmental Disorder (ARND) individuals present a higher tendency to alcohol and other drug problems (abuse or dependency) (Jacobs et al., 2000; Marmorstein et al., 2009). Thus, it was a completely unexpected result that

embryonic exposure to 0.25% and 0.5% alcohol did not show place preference after alcohol exposure at 10 or 90 dpf, while control fish spent more time in the area associated with alcohol exposure during the CPP test. It is not clear why ARND fish showed a different response, however, some hypotheses could be risen: we believe that the lack of association (place-drug) can be linked to embryonic alcohol exposure due to its effects on sensory processing and cognition.

Embryonic alcohol exposure is known to provoke a condition of under sensitivity to an environmental stimulus (Ornoy & Ergaz, 2010) and sensory integration deficits (Jirikowic et al., 2014; Muralidharan et al., 2015) that lead to inappropriate perception and response. Additionally, the cognitive loss has been documented for ARND (May et al., 2013; Murawski et al., 2015), and thus, zebrafish previously exposed to 0.25% and 0.5% alcohol may have presented difficulty in learning to associate US-CS. Studies in mice that fail to access CPP response discussed the results based on excessive stress related to alcohol administration (injection) and the drug effects on motor capabilities (Cunningham et al., 2002; Cunningham & Prather, 1992). In the present study, locomotion was barely affected, and alcohol administration followed a noninvasive protocol (alcohol dissolved into the tank water). In zebrafish, CPP failure has been related to alcohol concentration and exposure regime in adults, in which 0.1% alcohol did not cause CPP response while chronic exposure to higher concentration affected the response due to tolerance development (Chacon & Luchiari, 2014). As a result of control zebrafish (embryonically exposure to 0.0% alcohol) showed CPP at 10 and 90 dpf, we believe that the main effect on the other groups was the embryonic exposure to alcohol, which affected either the perception or the learning of the task.

Another unexpected result is related to the preference between white and striped sides of the arena. At 10 dpf, E0.25 changed side preference irrespective if exposed to 0.0% or 0.5% alcohol (Figure 2b), while E0.5 did not show side preference change after being exposed to 0.0% or 0.5% alcohol (Figure 2c). On the contrary, at 90 dpf E0.25 did not present side preference after exposed to 0.0% or 0.5% alcohol (Figure 2e) and E0.5 changed place preference both after 0.0% and 0.5% alcohol exposure (Figure 2f). Thus, the same embryonic alcohol treatment leads to different results in fish tested at the fry or adult stage. This result demonstrates that CPP response at larval stages and adulthood is not equivalent and raises questions regarding why the same alcohol concentration leads to different effects at different ontogenetic phases. Redesigning our protocol to test different cues instead of stripes and extending the alcohol association period (more than only one day) will be needed to clarify the specific reason for such a difference. Similar inconsistent results between various ages were observed in the study by Fernandes and colleagues (Fernandes et al., 2019), in which the authors observed embryonic alcohol exposure disruptive shoaling response in adults but not in larvae zebrafish. The authors refer to neurocircuitry differences between larval and adult social behaviors, which can also be a plausible explanation for the results obtained in our study on CPP.

As for the behavioral parameters related to locomotion and anxiety in fry zebrafish, no differences were observed for swimming speed and distance traveled, but immobility time was lower for E0.0F0.0 (control) than for E.0F0.5, E0.25F0.0, and E0.25F0.5 (Figure 3d). For adult fish, swimming speed and distance traveled were not statistically significant, and again immobility was higher for E0.5A0.05 than for control E0.0A0.05 (Figure 4d). These results suggest that the alcohol concentrations applied were not enough to impair fish locomotion at any ontogenetic stage, but strong enough to induce fish anxiety response. Freezing is a behavior phenotype commonly measured to indicate heightened anxiety (Blaser & Rosemberg, 2012; Egan et al., 2009). The increase in anxiety-like behavior on the day after the drug exposure may indicate a measure of abstinence, reinforcing our hypothesis that embryonic alcohol exposure may be related to an increased propensity to drug-seeking (Amorim et al., 2017; Cachat et al., 2010). Much has been shown about the harmful effects of embryonic alcohol exposure (Arenzana et al., 2006; Bilotta et al., 2004; Buske & Gerlai, 2011; Fernandes et al., 2018; Lovely et al., 2016), some of them related to drug use and abuse susceptibility in ARND individuals at the adult stage, but still nothing at younger phases.

5 | CONCLUSION

Our results show that zebrafish shows alcohol associative learning both at 10 and 90 dpf, which indicated the strong and reinforcing effect of the drug after a single exposure. However, embryonic alcohol exposure disrupts this response probably due to its harmful effects on brain areas related to perception and cognition that are extremely sensitive during embryogenesis. Future studies testing the other conditioning procedures as well as characterizing the brain areas affected by the embryonic alcohol exposure would be of great help to the thorough understanding of ARND. Nevertheless, the zebrafish as other relevant animal models are of extreme importance to understand which factors contribute to drug-seeking behavior, which are some other drug consequences to one´s life and may facilitate prevention and treatment strategies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS APPROVAL STATEMENT

All experiments performed here were conducted according to the guidelines of the Committee for Animal Use of Federal University of Rio Grande do Norte under the protocol CEUA 004002/2017.

AUTHOR CONTRIBUTIONS

JPS designed and performed experiments, analyzed and interpreted data, and wrote the article. HAS performed experiments, analyzed, and interpreted the data. ACL designed the experiments, interpreted data and wrote the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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