Long Term Effects of Early Embryonic Ethanol Exposure, on Behavioural Performance and Learning in Zebrafish, *Danio rerio*

By

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A thesis submitted in conformity with the requirements for the degree of Master of Arts
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Abstract

**Background:** Fetal alcohol syndrome (FAS) is a devastating disorder whose mechanisms may be best investigated using animal models. Here we present a novel zebrafish FAS model to investigate the effects of low to moderate alcohol exposure during early development on learning.

**Methods:** At 24-hours postfertilization zebrafish embryos were exposed to low doses of ethanol (external concentrations = 0.00, 0.25, 0.50, 0.75 and 1.00% vol/vol) for a very short duration (2 hours). Upon adulthood associative learning in the zebrafish was tested in a plus maze.

**Results:** This exposure led to no gross anatomical abnormalities or increased morbidity or mortality. Overall activity was not significantly affected by embryonic ethanol exposure. A trend towards a dose-dependent decrease in learning and memory performance was observed.

**Conclusions:** We suggest that zebrafish will be an appropriate model with which one can analyze the behavioural effects of embryonic alcohol exposure and the mechanisms of the ensuing abnormalities.
Acknowledgments

Many hands have helped to pave the road to my success.

I would like to thank my parents for supporting me throughout my success and my failures, for putting me first before themselves, for teaching me to always chase my dreams and for allowing me to chase them. I would like to thank my sister for picking up all the slack I have left behind and for all the help she has been to me. I would like to thank my Mindy for doing so much for me that words fail to quantify and express my eternal gratitude for you. I would like to thank my family and friends for their understanding and support. I would also like to thank Dr. Chatterjee for all his help. Finally I would like to thank Dr. Robert Gerlai, for giving me the opportunity to realize my dream and giving me the support and guidance to achieve it.
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1. Introduction

1.1 What is Fetal Alcohol Syndrome (FAS)? How does it affect human beings?

Maternal alcohol consumption during pregnancy can cause irreversible damage to the developing fetus hampering not only the individual’s life but also bearing a high cost to society. Individuals afflicted by aberrations caused by prenatal alcohol exposure (PAE) may require extensive family and social support in the areas of general health care, education, job training, justice and drug abuse (Stratton et al. 1996; Rasmussen et al., 2008). Fetal alcohol spectrum disorder (FASD) is an umbrella term used to describe any individual that has some physical, mental, behavioural or learning disabilities due to prenatal alcohol exposure. In Canada the estimated annual cost of care for those younger than 21 years of age suffering from FASD is $344,208,000. FAS is also the leading cause of preventable mental retardation and affects approximately 1 per 100 births (Rasmussen et al., 2008).

The most severe affliction on the spectrum is fetal alcohol syndrome (FAS) which was first described in the United States in 1973 by Jones and Smith after conducting case studies on infants born to mothers who were chronic alcoholics (Jones and Smith, 1973). Jones and Smith discovered characteristic reoccurring traits in these infants including facial abnormalities such as a flat upper lip, flat mid face, short nose, short palpebral fissure, and smooth philtrum; pre and/or postnatal growth retardation and neurodevelopmental abnormalities that include small cranial size at birth or structural abnormalities such as cerebellar hypoplasia, partial or complete agenesis of the corpus callosum or microcephaly, attention deficits, hyperactivity, learning problems, cognitive deficits, and/or motor problems; (Jones and Smith, 1973; Kodituwakku, 2007; Stratton et al., 1996). Jones and Smith’s ground breaking report served as the scaffolding
for following studies aimed at delineating the effects of PAE on the developing fetus. FAS is the most severe outcome of PAE, however milder cases on the spectrum may be more prevalent; i.e., cases in which affected individuals show no obvious physical malformations but have behavioural and cognitive deficits (McGee and Riley, 2007). Mild to moderate PAE in the clinical population can hamper a number of cognitive areas such as intelligence, executive functioning (EF), and language without causing gross morphological defects (for review see Kodituwakku, 2007; Rasmussen et al., 2008).

Learning and memory are also hampered by PAE (Kodituwakku, 2009). Streissguth et al (1990) explored the following question: Do the effects of PAE on learning still persist after seven years? To answer this question the researchers used multiple tests including the Wechsler Intelligence Scale for Children- Revised (WISC-R) and the Wide Range Achievement Test – Revised (WRAT-R). They also used questionnaires from parents and teachers to assess learning problems (for more details about tests see Streissguth et al., 1990). Based on the results of these tests and questionnaires Streissguth et al. (1990) concluded that children exposed to PAE suffered from learning problems even after seven years. Hamilton et al. (2003) used a virtual Morris water maze task to examine the affects of PAE on place learning and cue navigation in eight adolescent males with FAS and eight controls with no history of PAE. In this study, children learned to navigate to a hidden platform in a fixed location of a virtual pool. Each child conducted 20 hidden platform trials, after which a single no-platform probe trial was administered. Eight cue-navigation trials followed this task during which the platform was visible. Hamilton et al. (2003) found that children with a history of PAS performed more poorly on the hidden trials and the probe trials than controls and thus concluded that the FASD group demonstrated deficits in spatial learning. In a study conducted by Mattson et al. (1996), the
California Verbal Learning Test – Children’s version was used to assess components of learning and memory in children with FAS. The authors reported that children with a history of PAE had difficulty learning and recalling verbal information. Furthermore, as part of their 16 year longitudinal study Willford et al. (2004) used the Children’s Memory Scale (CMS) to assess learning and memory in adolescent that were prenatally exposed to moderate levels of alcohol. These authors also found that impaired performance on global measures of learning and memory were predicted by PAE. Thus all the aforementioned studies demonstrate that PAE affects learning and memory in humans.

1.2 Does PAE affect learning in animals?

Rodent models have primarily been used to study the effects of PAE on learning and memory (Kodituwakku, 2009). Various authors using several pieces of apparatus have demonstrated that spatial learning and memory in rodents are hindered by PAE (for full review see Berman and Hannigan, 2000). Lochry and Riley (1980) were the first researchers to report dose-dependent increase in errors on a T-maze in rats that were prenatally exposed to alcohol, showing that deficits in learning and memory are associated with PAE. Wainwright et al. (1990) also demonstrated that PAE affects learning, but used mice as their animal model. Mice were required to learning the location of a hidden escape platform in a water filled T-maze. In order to reach criterion the animal had to complete 5 consecutive trials without an error. After reaching criterion the escape platform was switched to the opposite arm requiring the subject to reverse their previous learning. Wainwright et al. (1990) found that rats with PAE demonstrated deficits in learning.
The Morris Water Maze (MWM) is a classic test of spatial learning in which rodents placed in a shallow pool of opaque water and are required to use visual cues to learn the location of a submerged escape platform (Sweatt, 2010). Gianoulakis (1990) used the MWM to evaluate whether in utero alcohol exposure hampered the learning process in Sprague-Dawley rats at 40, 60 and 90 days of age. Gianoulakis (1990) found that rats prenatally exposed to alcohol took longer to perform the swim task, took longer to search for the removed platform and swam longer distances to find the platform, thus demonstrating that PAE hampers learning and memory.

1.3 Have zebrafish been used to study FAS?

In the past, zebrafish have been utilized as a model of FAS (Bilotta et al., 2004; Carvan III et al., 2004; Arenzana et al., 2006; Matsui et al., 2006; Fernandes & Gerlai, 2009). Bilotta et al. (2004) found that zebrafish embryos exposed to ethanol developed microphthalmia (decreased eye size and increased distance between the eyes), exhibited heart rate abnormalities, enlarged body cavities, smaller standard body length and higher mortality rates. Carvan III et al. (2004) found that PAE led to increased cell deaths in the CNS; craniofacial and skeletal deformities; notochord and spinal chord developmental abnormalities; and altered gene expression in the fore- and midbrain. Arenzana et al. (2006) found that zebrafish with a history of PAE had cyclopia (fusion of two eyes) along with other numerous cytoarchitectural abnormalities. Matsui et al. (2006) found morphological and functional abnormalities of visual system of zebrafish with PAE.

The studies by Bilotta et al. (2004), Carvan III et al. (2004), Arenzana et al. (2006) and Matsui et al., (2006) share some common factors. All of them used high concentrations of
alcohol and/or long exposure to this substance. For example Bilotta et al. (2004) varied the start of PAE, beginning at 0 hours post fertilization (hpf) up to 48 hpf, while exposing the zebrafish embryos to 0, 1.5 or 2.9% ethanol on average for 18.3 hours; with the minimum exposure time being 8 hours and the maximum exposure time being 24 hours. Arenzana et al. (2006) treated their zebrafish embryos with 1.5 or 2.4% alcohol starting at 4.7 hpf and continued the alcohol treatment until 24h post fertilization. Arenzana et al. (2006) used concentrations ranging between 1.0 and 2.4% to treat zebrafish embryos from 4.3 hpf to 24 hpf. Matsui et al. (2006) used slightly lower range of alcohol concentrations (1 to 2%) however the researcher exposed the zebrafish to 72 hours of alcohol, beginning at 48 hpf up to 120 hpf. Like Matsui et al. (2006) Carvan III et al. (2004) also exposed their zebrafish embryos to long bouts of alcohol; these researchers exposed their fish to alcohol from 4 hpf up to 6 days (144 hpf). While these studies were important in providing evidence that the zebrafish respond to embryonic alcohol treatment, they represented circumstances that were highly extreme and perhaps extremely rare in human. Briefly, alcohol consumption by pregnant women is rarely associated with such high levels of, or prolonged exposure to, alcohol in the fetus (e.g. see discussion of this topic in Matsui et al., 2006; also see Fernandes & Gerlai, 2009). These studies also failed to investigate the behavioural consequences of PAE.

1.4 Can zebrafish learn?

A number of different authors have demonstrated that zebrafish can learn (Bilotta et al., 2005; Blank et al., 2009; Braubach et al., 2009; Eddins et al., 2009; Hicks et al., 2006; Xu et al., 2007; Pather and Gerlai, 2009; and Sison and Gerlai, 2010). Bilotta et al. (2005) taught zebrafish to use a visual cue to make an appetitive choice discrimination whereas Braubach et al. (2009)
used an olfactory cue towards the same end. Hicks et al. (2006) demonstrated that an automated learning paradigm can be used to teach zebrafish, while Xu et al. (2007) used a shuttle box to successfully teach zebrafish to avoid an electric shock, thus demonstrating that zebrafish can learn avoidance responses. Like Xu et al. (2007), Pather and Gerlai (2009) used a shuttle box to demonstrate that zebrafish can learn however instead of using an electric shock, these researchers used a computer image of a zebrafish shoal.

Sison and Gerlai (2010) used a plus maze to examine whether zebrafish could learn a simple associative tasks as well as a spatial learning task. In their study Sison and Gerlai (2010) trained zebrafish to associate a red cue card with a food reward (simple associative task) and to use spatial cues from around the testing room to find the fixed location of the cue card. The authors had 2 groups: Paired and Unpaired. In the simple associative learning task the Paired group had a red cue card placed beside the feeding tube while the Unpaired group had a red cue card placed in area that was not beside the feeding tube. In the spatial learning task, fish were required to learn the fixed location of the food using extra-maze cues. The location of the food was kept constant during the time the Paired group was trained, therefore making it possible for the zebrafish to use the various extra-maze cues to learn the location of the food. The Unpaired group had food placed in random locations. Sison and Gerlai (2010) found that the Paired group spent more time in the target arm, during the simple associative task and the spatial learning task compared to the Unpaired group thus demonstrating that zebrafish can learn a both a simple associative task and a more complex associative spatial learning task.
1.5 Why use zebrafish to study the effects of FAS on learning and memory?

In the area of FAS research, zebrafish are a relatively new species that have been underutilized. Nevertheless, this species has already demonstrated a promise as a potential model (Fernandes & Gerlai, 2009). Over the past thirty years research on zebrafish has burgeoned in developmental biology to the point that zebrafish have now become one of the three preferred laboratory species in genetics (Grunwald & Eisen, 2002). Numerous genetic tools have been developed for this species and a large amount of genetic information (e.g. genetic markers, sequencing of its genome, etc.) has been accumulated (Grunwald & Eisen, 2002). In addition to its strong genetics, the zebrafish has a number of advantages as a model organism (Gerlai et al., 2000). Zebrafish are small (3 – 4 cm) and robust fish that breed throughout the year and can be easily and cheaply housed in the laboratory. They yield a large number of eggs; every other day a female zebrafish can produce 200 eggs (Nusslein-Volhard & Dahm, 2002). Zebrafish have a short generation time and rapid development, taking typically 3 to 4 months to go from eggs to sexually mature adults (Nusslein-Volhard & Dahm, 2002; Gerlai et al., 2000). Moreover, a number of zebrafish genes have already been shown to be evolutionarily conserved. According to Lockwood et al. (2004) the nucleotide sequence of zebrafish genes is 70-80 percent similar to that of mammalian, including human genes. The zebrafish have a complex set of behaviors (Gerlai et al., 2000). Moreover, since fertilization and embryonic development occur externally (Nusslein-Volhard & Dahm, 2002) and alcohol diffuses across the shell of the egg into the embryo, the complicating effects of placental function or the physiology of the mother are absent. Also, the effects of acute as well as chronic alcohol exposure on zebrafish have started to be characterized (Gerlai et al., 2000; Dlugos & Rabin,
2003; Lockwood et al., 2004; Gerlai and Blaser, 2006; Gerlai et al., 2008). In summary, the combination of all these factors makes the zebrafish a good animal model for analyzing effects of PAE.

1.6 Have zebrafish been used to study the effects of PAE on learning?

While there have been numerous rodent studies addressing the issue of FAS and learning (see above), there currently is only one other study that has addressed this issue using zebrafish. Carvan III et al. (2004) exposed zebrafish embryos to high concentrations of ethanol from 4 hpf up to 6 days post-fertilization and measured a number of physiological and behavioural measures, including learning. These authors divided a tank with an opaque divider that allowed the fish to swim under it, in order to reach the other side of the tank. Initially food was presented on the same side as a red cue, and then later food was presented on the opposite side of the cue card. Fish were to reverse their previous learning (red card means food). These authors found that learning was hampered in a dose-dependent manner when zebrafish embryos were treated with a low dose of ethanol (Carvan III, 2004).

According to Bilotta et al. (2004) zebrafish provide a number of advantages over rodent models when studying FAS or PAE. The precise control over the experimental conditions is the most significant advantage of the zebrafish. The concentrations across each embryo stay constant, since the alcohol diffuses directly from the water into the egg, thus removing the confound of maternal physiology that plagues rodent models (Bilotta et al., 2004). Since fertilization and embryonic development occur externally (Nusslein-Volhard & Dahm, 2002) the complicating effects of placental function or the physiology of the mother are absent (Bilotta et
al., 2004). Furthermore, the timing of the exposure is precise, when using zebrafish since the eggs can be rinsed clean of any alcohol. Also, maternal rearing is not an issue with zebrafish since the mother is not present during rearing (Bilotta et al., 2004). As mentioned by Sison and Gerlai (2010) in the past the tools available to test learning and memory in zebrafish was wanting, however as demonstrated above, currently there are multiple studies supporting the claim that zebrafish can learn.

We chose to explore learning and memory after embryonic alcohol exposure. However, in our study we exposed zebrafish to a range of alcohol concentrations (0, 0.25, 0.50, 0.75 to 1.00% vol/vol) which was lower and was for a shorter period (for 2h at 24 hpf) than what was employed in other laboratories (Bilotta et al., 2004; Carvan III et al., 2004; Arenzana et al., 2006; Matsui et al., 2006).

The goal of the current study is to investigate whether this embryonic alcohol exposure regimen, which is more realistic in terms of clinical relevance, may lead to impaired learning performance in zebrafish when tested at their fully mature adult stage. Interestingly this embryonic alcohol exposure regimen we have found to impair social behaviours in adult zebrafish in a dose-dependent manner, i.e. as the embryonic alcohol concentration increased social behaviour in the adult decreased (Fernandes and Gerlai, 2009). While most of the past research has used high concentrations of ethanol for long periods of time (Bilotta et al., 2004; Carvan III et al., 2004; Arenzana et al., 2006; Matsui et al., 2006), I was hoping to emulate the subtle behavioural changes present in FASDs without the gross structural and morphological changes observed in FAS. Another reason the current dosing regime was chosen, was because according to Fernandes and Gerlai (2009) no gross morphological or structural abnormalities were observed, yet social behaviour was impaired.
To measure learning and memory in zebrafish exposed to PAE, we used a plus maze employed by Sison and Gerlai (2010). The current study is based on the design of Sison and Gerlai (2010) but numerous physical as well as procedural characteristics of the task were modified. For example, I do not have paired and unpaired groups but rather 5 treatment groups, the different embryonic alcohol concentration groups, where the doses and exposure regimen were based on my past research (Fernandes and Gerlai, 2009; also see below). Instead of using syringes filled with Gelly Belly fish food as employed by Sison and Gerlai (2010) I used TetraMin tropical food flakes presented in a floating red Plexiglas doughnut. The particle size of this food can be kept uniform, which better controls the size of reward, and presentation in the floating red Plexiglas doughnut better replicates how zebrafish would forage in nature (zebrafish mainly eat small insects fallen into the water). Furthermore, instead of placing red cue cards beside the food I used a red Plexiglas doughnut (the ring that contained the food) which was suspended by an aluminum rod hooked to the maze. This allowed me to float the food in the center of the doughnut and to teach the fish that the red Plexiglas doughnut is associated with a food reward. Based on the findings of Carvan III et al. (2004), Lochry and Riley (1980) and Fernandes and Gerlai (2009) I hypothesize that there will be a dose-dependent decrease in learning, i.e., the 1.0% will spend the least time in the target zone (near the red doughnut) and will also visit the target zone less frequently compared to controls.

In the current study I exposed zebrafish embryos to 5 concentrations of alcohol, ranging from 0 to 1.00% for only 2 hours at 24 hours postfertilization. I measured the potential effects of the manipulation in adult zebrafish (1 ½ year-old) by using an event recording software application, Observer Color Pro (Noldus Info Tech, Wageningen, The Netherlands), to quantify the time the fish spent in different areas of the maze and the frequency of the visits the fish made
to these areas including the target zone in which the food reward was presented (see below). I hope that by establishing a zebrafish model for FASDs, I can eventually facilitate the characterization of the molecular and neurobiological mechanism underlying the learning and memory deficits observed in children whose mothers consumed small to moderate amounts of alcohol during pregnancy.
2. Methods

2.1. Experimental Subjects

100 Zebrafish embryos were obtained from our own vivarium (UTM). Embryos were collected approximately 2.5 hours post fertilization (hpf). Embryos were then divided into 5 equal groups and placed in 100 ml Petri dishes. Five different concentrations of ethanol were created: 0 %, 0.25 %, 0.50% 0.75% and 1.0% diluting purified 99.99% Ethanol with system water (the water reverse osmosis purified and 60 mg/l Instant Ocean Sea Salt reconstituted water that is used for the maintenance of our zebrafish). Each zebra fish group was treated with one concentration of ethanol 24 hpf, a between subject experimental design. Zebrafish were exposed to the ethanol for 2 hours by placing them in 100 ml of the corresponding ethanol solution in 100 ml Petri dishes. Subsequently, the embryos were removed from the solution, and placed in 100 ml of system water in new 100 ml Petri dishes. The zebra fish spent 5 days in the Petri dishes before being moved to 2.8 l Plexiglas holding tanks. After spending 3 weeks in the holding tanks the zebra fish were moved to 2.8 l Plexiglas aquaria designed by Aquaneering Inc. (San Diego, CA, USA) which were part of a recirculating filtration aquaculture rack system designed specifically for zebra fish. All fish were kept in the same described reverse osmosis purified and salt reconstituted system water from hatching to adulthood. A 12 hour light / 12 dark cycle was maintained with the fluorescent lights turning on at 7 am and turning off at 7 pm. All fish were fed twice a day with ground freeze-dried krill and flake food (TetraMin Tropical Flakes, Tetra. USA).
2.2. Apparatus and procedure

The test apparatus (Figure. 1) was a four-arm, plus-shaped transparent Plexiglas maze similar to that employed by others (Sison and Gerlai, 2010; Salas et al., 1996). Each arm of the maze was 35cm long, 11cm wide and 20cm high. The arms were connected to each other by an 11×11cm center square into which a start box could be lowered and could be made accessible by lifting the start box. At the end of each arm was an 11 cm long Plexiglas rod which was glued across the width of the arm. The cue used was a red doughnut shaped Plexiglas disc that had an outer diameter of 3.8 cm and a hollow center of 2 cm. The red Plexiglas doughnut was attached to a 13.5 cm aluminum rod that had hooks on both ends. One end of the aluminum rod hooked the Plexiglas doughnut to the rod while the other end hooked on the 11cm Plexiglas rod of the maze, thus allowing the red Plexiglas doughnut to stay atop the water and also stay at the end of the arm. The food reward used was TetraMin tropical flake food. I used a punch that was 3 mm in diameter to make 3 mm disc of TetraMin food. The maze was surrounded by a plywood box that was 122cm X 102cm and was used to prohibit the fish from using external spatial cues to locate the cue. A JVC Everio HDD camera was mounted above the maze to record the behaviour.
2.2.1. Habituation trials

To acclimatize and shape the fish to the maze, they were administered four habituation trials (30 minutes every other day for 4 days). During the 30 minute habituation trials all arms of the maze were baited (i.e. access to the food reward was allowed) with TetraMin tropical flakes (food reward) that were 3 mm in diameter. We used this approach to acclimatize our fish to the maze and to make them learn that food is present in the maze (procedural learning and shaping). Food was placed in the center of the red Plexiglas doughnut. Fish were only fed when they were in the maze to avoid satiation.
2.2.2 Simple associative learning: training

On completion of habituation fish began training which lasted for 5 days. Fish were deprived of food 24 hours before each training day. Unlike the habituation phase, during the training phase the red Plexiglas doughnut was only placed in one arm. Fish were placed in a start box in the center of the maze. The trial began once the start box was lifted and the trial lasted for five minutes. Each fish was trained 4 times a day during between 11:00h and 19:00h with the cue being placed in a different arm for each trial. For all fish the food rewarded arm changed randomly from trial to trial. For each trial, fish were removed individually from their holding tank and transferred into the centre start box of the maze, where they acclimatized for 10 seconds. The box was then lifted using a 6ft rod with a hook on the end. In summary, fish received a total 20 training trials before the probe trial that tested their acquisition of memory (please see below). Each session was videotaped and the recordings were later replayed for analysis.

2.2.4. Probe trial

The probe trial is the most important part of this behavioural procedure as it allowed us to quantify acquisition of the association between cue and reward. During the probe trial only the conditioned stimulus, the red Plexiglas doughnut, was presented, and all other procedures were the same as in the training trials except that no food was accessible in any arm of the maze. The length of the probe trial was also 5 min.
2.2.5 Quantification of behaviour and statistical analysis

The location of fish was measured using the event recording software application Observer ColorPro 5.0 (Noldus, Info Tech., Wageningen, The Netherlands). We quantified the percent of time the fish spent in the target zone (figure 1), in the center square, and in the other arms of the maze during the probe trial. We also recorded the number of times the fish entered the target zone and other zones of the maze during the probe trial. We statistically analyzed the following measures: Percent of time the fish stayed in the target zone and the number of visits to this zone, measures that may reflect the strength of preference for and thus perhaps memory of the conditioned stimulus (the visual cue associated with the food reward); The number of visits to all zones was also analyzed as it quantifies general locomotor activity, an important component of an active appetitive learning task;

Due to the small sample size and difficulty of gender determination the data were pooled for gender. Data were analyzed using SPSS (version 14.1) for the PC. Repeated measure variance analysis (ANOVA) was used to investigate the effect of interval (five 1min intervals, the repeated measure factor), the effect of alcohol treatment (5 doses, the non-repeated between subject factor), and the interaction between these factors. Non-repeated measure ANOVA was also used for measures derived from the interval data (the total duration of time spent in particular zones during the probe trial). Where appropriate, one sample T-tests were used to compare performance to chance. Chance performance was calculated assuming equal distribution of time spent in all areas of maze (for example chance level period of time spent in N1 zone = surface area of N1 divided by total surface area of the maze times duration of probe trial). The Bonferroni correction was used to correct the α level to 0.01 (p = 0.05 divided by 5;
because I made 5 comparisons) where multiple comparisons were necessary. This was done in order to control for the overall Type I error within the experiment (Field, 2005).
3. Results

No physical abnormalities were observed in the developing or adult fish exposed to alcohol. A univariate analysis of variance of the total activity (Total Number of Zone Visits) (Figure 2) revealed that Early Embryonic Ethanol Exposure had no significant effect on total activity as measured by the total number of entries to zones ($F(4, 55) = 1.087, p = .37$). The mortality rate of the exposed fish was also not increased.

![Figure 2](image.png)

*Figure 2.* Total number of visits to all zones during the probe trial. Mean±SEM are shown.

Although there is an apparent trend observable in figure 3 suggesting a potential impairment of probe trial performance in fish treated with embryonic alcohol exposure, ANOVA revealed a nonsignificant main effect of Interval on the time spent in the N1 ($F(4, 216) = 1.84, p = .12$) suggesting that the percentage of time spent in the target zone during the probe did not vary across different intervals (figure 3). Furthermore, ANOVA also revealed a nonsignificant interaction effect between Interval x Alcohol treatment ($F(16, 216) = .55, p > .05$) on time spent in N1, suggesting that alcohol treatment did not affect the time spent in the target zone in an interval dependent manner. ANOVA also revealed a nonsignificant Alcohol treatment effect ($F$
(1, 54) = .605, p > 0.05), showing that the time spent in the target zone did not vary across Alcohol treatment groups.

To further analyze the data we also compared the time spent in N1 to random chance, and we also analyzed the time spent outside of N1 and compared it to chance. Figure 4 shows that the time spent in N1 (target zone) during the probe trial during the probe trial was also nonsignificant (F (4, 59) = .662, p>.05). Next, the duration of time fish spent in the target zone was compared to random chance for each of the five groups (random chance corresponds to 4.64 sec for the target zone, a dwell time value that is calculated on the basis of the area of the target zone relative to the total area of the maze as explained above). Performance comparisons of the control group to random chance (μ = 4.64) showed that the average time the Control group (M =
15.35, SD=18), spent inside of the target zone although did not differ significantly from chance ($t(10) = 1.97, p = 0.078$) the obtained p value bordered significance. Performance comparison of the 0.25% alcohol group (M = 12, SD = 17.02), to random chance ($\mu = 4.64$) showed that the average time spent in the target zone did not significantly differ from chance ($t(10) = 1.97, p = .16$). The 0.50% alcohol group (M = 14.31, SD = 14.76 ) showed a trend towards significantly spending more time inside of the target zone compared to random chance, ($t(11) = 1.50, p = .06$). Performance comparisons of the 0.75% alcohol group (M= 8.74, SD = 8.63) and the 1.0% alcohol group (M = 7.81, SD = 10.22) showed that the averaged time spent inside the target zone did not significantly differ from chance, ($t(10) = 1.58, p = .15$; $t(10) = 1.16, p = .27$).

![Figure 4](image)

**Figure 4.** Duration of time spent in N1 (the target zone) during the probe trial. Mean±SEM are shown.

Next in order to examine if the fish spent more time outside of the N1 (target zone) but inside of the target arm (North arm) (i.e. not in the closest proximity to the conditioned stimulus but at least in the arm that contained this stimulus) I averaged the time the fish spent in the N2 and N3 (the two zones outside of the target zone but still within the target arm, see figure 1) during the probe trial. Figure 5 suggests that the average time spent outside the target zone during the probe trial also did not differ across treatment groups, an observation confirmed by
ANOVA that revealed a nonsignificant alcohol treatment effect (F (4, 59) = 1.421, p>.05). Performance comparisons of the groups to random chance showed that the average time of the Control group (M = 1.91, SD = 1.91) spent outside of the target zone but within the target arm was significantly below chance (µ = 4.64) (t (10) = -4.65, p = 0.01). The average time of the 0.25% alcohol group (M = 2.82, SD = 2.28) spent in the target arm outside of the target zone trended below chance (4.64), (t (11) = -2.56, p = 0.019). However the average time of the 0.50% alcohol group (M = 6.66, SD = 7.22), the 0.75% alcohol group (M = 6.23, SD = 7.6) and the 1.0% alcohol group (M = 6.60, SD = 9.29) spent in the target arm outside of the target zone did not significantly differ from chance (µ = 4.64), (t (10) = -1.20, t (10) = -1.33, t (13) = -1.10, p > .05, p > .05, p > .05)

Figure 5. Average duration of time in the target (North) arm outside of the N1 zone during the probe trial. Mean ± SEM are shown.

Above I first focused on the North arm as it is the target arm that contained the associative cue. Below I briefly discuss the time the fish spent in other areas of the maze. . Figure 6 shows the time fish spent in the E1, W1 and S1 zones (the end of the non-target arms) and also the time spent in the E, W and S arms outside of the end zones E1, W1 and S1. ANOVA revealed that there was no significant effect of Alcohol treatment on the time spent in the East
arm end zone (E1) \( (F (4, 54) = .505, p = .733) \), the West are end zone (W1) \( (F (4, 54) = .734, p = .570) \) or the South arm end zone (S1) \( (F (4, 54) = 1.23, p = .306) \). ANOVA also revealed that there was no significant effect of Alcohol treatment on the time spent in the East arm outside of the end zone \([(E2+E3)/2] (F (4, 54) = .221, p = .925)\), in the West arm outside of the end zone \([(W2+W3)/2] (F (4, 54) = .166, p = .179)\), or in the South arm outside of the end zone \([(S2+S3)/2] (F (4, 54) = .271, p = .896)\).

**Figure 6.** Average duration of time fish spent in the E1, W1, or S1 end zones (the end of the corresponding arms in which no associative cue was present), and duration of time fish spent in the no cue arms (E, W, and S) outside of their end zones during the probe trial. Mean ± SEM are shown.
I also calculated the time the fish spent in the center of the maze during the first 5 minutes of the probe trial. ANOVA revealed a nonsignificant main effect of Alcohol treatment on the time spent in the center ($F(4, 54) = 0.626, p > .05$) (Figure 7). Performance comparisons of the time each group spent in the center compared to random chance ($\mu = 4.64$) were nonsignificant; Control ($M=9.72, SD = 16.07), (t (10) = .32, p =0.32$); 0.25 group ($M = 4.64, SD = 3.48), (t (11) = 0.001, p = .99$); 0.50 group ($M=5.27, SD = 2.62), (t = .80, p = .45$); 0.75 group ($M = 10.31, SD = 12), (t (10) = 1.61, p = .138$) and 1.0 group ($M = 8.78, SD = 14.18), (t (13) = 1.10, p = .294$).

![Figure 7](image-url)

**Figure 7.** Duration of time fish spent in the center during the probe trial. Mean ± SEM are shown.

Figure 8 shows the frequency of entries fish made into the target zone during the 5 minute probe trial. ANOVA revealed a nonsignificant Interval effect ($F (4, 216) = 0.96, p > 0.05$) suggesting that the number of visits to the target zone during the probe did not vary across different intervals. Moreover as also evident from Figure 8, there was a nonsignificant interaction between Interval x Alcohol treatment ($F (16, 216) = 1.24, p>.05$), therefore demonstrating again that alcohol treatment did not affect the number of visits to the target zone in an interval dependent manner. However, ANOVA revealed a trend towards a significant
Alcohol treatment effect ($F(4, 54) = 2.42, p = 0.078$), suggesting that the frequency of visits to the target zone may vary across different alcohol treatments.

**Figure 8.** Total number of visits to N1 during the probe trial. Mean ± SEM are shown.
4. Discussion

Fetal alcohol syndrome is the leading cause of preventable mental retardation in the Western World that not only costs society an astronomical amount of tax dollars, but more importantly it causes an individual to have a lifelong disability that reduces their quality of life (Eustace et al., 2003). On the continuum of negative effects caused by PAE, FAS is the harshest outcome that is associated with heavy drinking (Kodituwakku, 2007). However, the consumption of moderate levels of alcohol (two standard drinks, per day) during pregnancy can also cause damage to the developing fetus, i.e., it can cause subtle behavioural and/or cognitive deficits without causing any gross morphological changes that may be detectable only when the exposed children grow up (Redgrave et al., 2003). The goal of the current paper was to examine whether small concentrations of alcohol administered for a short period of time would lead to detectable deficits in associative learning in a plus-maze.

The first point to consider is whether the learning paradigm worked. The task presented here was a significantly modified version of a previous apparatus and procedure (Sison & Gerlai, 2010). Both the type of food and the manner in which it was presented was modified to improve the task. Most importantly, in the previous task zebrafish became satiated quickly and their motivation to perform in the task diminished after repeated trials (Sison and Gerlai, 2010). I did not observe such change in motivation (number of visits to the target arm during training); in this sense the modification was a success. However, examination of the performance of the control group suggested that although fish in this group tended to spend somewhat more time in N1 target zone, the area closest to the conditioned stimulus, in the probe trial, this performance only bordered significance but did not quite reach it, i.e., the task was not robust enough. This may be
due to inadequate number of training trials run or perhaps experimental error variation and or individual differences among the tested fish.

I examined the time the fish spent in each of the areas and compared them to chance in order to determine if these fish actively chose to be in any one given area compared to another. For example, if the control group spent more time in N1 (target zone) compared to chance, while spending less time outside of the N1 ((N2+N3/2)) or the center compared to chance, it would appear that the fish would have a preference for the target zone. While the average time spent in the N1 by the control group did not significantly differ from chance, there was a trend towards significance. The average time spent by the control group outside of the target zone was significantly less than chance. Furthermore, the average time the control fish spent in the center of the maze did not significantly differ from chance. Thus when all three of these results are viewed together it appears that zebrafish without a history of PAE did not swim completely homogeneously in all areas of the maze implying that these fish may have learned to associate the cue with the food reward. This finding would be in line with previous research by Sison and Gerlai (2010) who found that zebrafish that were paired with a cue spent significant more time in the target arm than chance alone compared to fish that were not paired with a cue.

A plausible reason for failing to replicate the findings by Sison and Gerlai (2010) could be due the variation in procedure that I used to measure learning and memory; which included changes in the type of food, delivery manner, test schedule and cue size. As mentioned earlier I decided to change the type of food used (Flake food versus Jelly Belly gel food) as well as the manner of delivery (floating flakes versus injected jelly) in order to avoid the problems of satiation and the subsequent decrease in motivation that occurred in the past work by Sison and Gerlai (2010). In their study these researchers tested fish on 3 consecutive days while I tested for
5 days, every other day. Furthermore, these researchers also used a bigger cue card (7 cm wide X 10 cm high) compared to the red Plexiglas doughnut (diameter = 4.8 cm) used in the current study. Thus it is plausible that the gap between training days and the smaller cue may have weakened the pairing in the current study, thus making it more difficult for the fish to learn.

Sison & Gerlai (2010) used a sample size of 10 fish per group and was the basis for my decision to set my per-group sample size around this number. In the current study each group on average had 11 fish, which however, may not be a large enough sample to detect a significant difference due to the changes in procedure and quantification mentioned above.

I believe, using TetraMin flake food is advantageous over the Gelly belly food used by Sison and Gerlai (2010) for a couple of reasons. First, according to the authors Gelly belly satiated the fish too fast, which could hamper learning, simply because if the fish is satiated in the first trial, they will not be motivated to search for food in the second trial. Secondly, according to the authors Gelly Belly food was difficult to deliver in a localized manner. The current study addressed these issues by using flake food. The flake food was uniform in size (3mm diameter circles) and floated in the center of the red Plexiglas doughnut. The flakes were also small enough to prevent satiation.

The current study was also successful in demonstrating that the dosing regimen used is suitable for creating zebrafish that do not have gross morphological or structural abnormalities; making this dosing procedure suitable for studies that require the generation of fish to model FASDs versus FAS. The finding that there was no significant Alcohol effect or Alcohol Treatment X Interval interaction effect on the frequencies of entries to the arms (Total Activity) supports the aforementioned point. This finding is supported by previous research conducted by
Fernandes and Gerlai, 2009 who used the same exposure time and concentrations and found a dose-dependent decrease in social behavior.

The problems mentioned earlier may be addressed in the future by increasing the sample size of the groups and/or by increasing the number of training trials, and/or by increasing the saliency of the Conditioned Stimulus (for example, by providing a more vivid colour or a larger surface area visual cue. Increasing the strength of the reward by making the fish hungrier is another possible way to improve the robustness of the task, working hypotheses whose validity will be ascertained in the future.
REFERENCES


