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The Development and Morphology of Zebrafish after Embryonic Ethanol Exposure

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Abstract

Maternal consumption of alcohol may subject the fetus to fetal alcohol syndrome or fetal alcohol spectrum disorders (FAS or FASD). FAS/D is a public health problem, and affected children are defined by varying degrees of irreversible mental retardation, physical defects, behavioral issues, and vision problems from prenatal alcohol exposure (Riley et al. 2011). Recent studies on FAS have looked towards animal models, such as zebrafish, *Danio rerio*, that exhibit homologous physical and behavioral effects of alcohol (Bilotta et al. 2004). I exposed zebrafish embryos to low doses of ethanol (0.5% v/v or 1% v/v) in either chronic (at least 8 h of exposure) or acute (1 or 4 h of exposure; up to four times before hatching) patterns at different points during development. I tested the hypothesis that the effects of the ethanol exposure on the morphology, function, and behavior of zebrafish vary depending on the exposure period and stage of development, and that the severity of physical, functional, and behavioral differences in ethanol exposed groups are related to ethanol concentration. The results show that embryonic exposure to low doses of ethanol indeed affects the morphology, function, and behavior of larval zebrafish. Embryos in chronic exposure treatments exhibited differences in morphology in a dose dependent and stage specific manner, and in physiology regardless of morphological differences. Embryos in acute exposure treatments exhibited differences in morphology in a dose and somewhat frequency dependent manner; physiology of these zebrafish was affected regardless of morphological differences. Results from one acute treatment group suggests there is a threshold at which low doses of ethanol for short durations or at specific stages of development would not physiologically or morphologically affect the zebrafish. Behavioral data on one chronic treatment group demonstrated increased preference for lit environments, which may reflect eye function.

Introduction

The term 'fetal alcohol syndrome' was first coined in 1973 when researchers found that children who were prenatally exposed to alcohol displayed a constellation of certain facial features, growth deficiencies, and cognitive or intellectual deficiencies (Jones and Smith, 1973, Clarren and Smith, 1978). Today, fetal alcohol syndrome disorder (FASD) is a national and global public health issue. FASD ranges in prevalence from 2 - 5% of children in the United States and Western Europe (May et al. 2009). In districts in South Africa with historically high risk drinking behavior, approximately 8.9% children are considered to have FASD, and the prevalence rate of FASD in all children in the Mediterranean region ranges from 2.3 - 4.1% (May et al. 2009).

FASD is well described as a spectrum disorder; children with the complete phenotype at the severe end of the spectrum are classified as having fetal alcohol syndrome (FAS). Individuals identified with FAS must present prenatal or postnatal growth deficiency, characteristic facial features, and central nervous system dysfunction (Streissguth et al. 1980, Wattendorf and Muenke 2005, Fryer et al. 2007). Prenatal and postnatal growth deficiency has been observed in individuals with FAS. Covington et al. (2002) reported that children at age seven who were prenatally exposed to alcohol and born to women over 30 were up to 14 pounds lighter and five times more likely to fall below the 10^{th} percentile in weight. Facial and cranial anomalies used to diagnose FAS include short palpebral fissures, thin vermilion border of the upper lips, flat nasal bridge and midface, epicanthal folds, underdeveloped upper ear, and small head circumference (Jones et al. 2010). Finally, central nervous system dysfunction may occur from prenatal ethanol exposure without either the growth deficiency or characteristic facial features (Fryer et al. 2007). Guerri et al. (2009) imaged the brain and analyzed behavior of children with FASD. They found that the central nervous system is vulnerable to the teratogenic effects of ethanol, and that brain

abnormalities can range in severity. In addition to these direct indicators, individuals diagnosed with FAS or FASD are at greater risk for other various chronic health issues. For example, one third of children prenatally exposed to alcohol also have congenital cardiac problems (Ornoy and Ergaz 2010). Even without the presence of congenital cardiac problems, prenatal exposure to ethanol can still lead to minor cardiac abnormalities (Krasemann and Klingebiel 2007).

Damage to the central nervous system can manifest into a variety of cognitive and behavioral problems. While many with FAS are diagnosed with mental retardation, others exhibit difficulties in learning, language, motor skills, visuospatial ability (e.g., depth perception and balance), and deficits in cognitive control (Fryer et al. 2007). They are also at risk for developing depression, substance use disorders, and antisocial personality traits (Fryer et al. 2007). Many children with FAS have severe psychopathological and behavioral issues that persist throughout adolescence and into adulthood, and are dependent on support from home, school, or society throughout their entire lives (Steinhausen et al. 1998). Furthermore, Sood et al. (2001) found that while all children exposed to alcohol prenatally were 3.2 times more likely to exhibit 'delinquent' behavior compared with children not exposed to alcohol, children exposed prenatally to low levels of alcohol (less than 0.3 fluid ounces of alcohol) demonstrated externalizing and aggressive behaviors.

There are many studies that seek to understand genetic factors behind FASD susceptibility (Olney et al. 2002). The results of these genetic epidemiological studies could reveal a correlation between increased susceptibility to FASD and genetic variation; past studies on twins and mice have pointed to the genetic risk for FASD (Streissguth and Dehaene 1993, Becker et al. 1996). Various alleles that code for alcohol dehydrogenases and thus influence the metabolism of alcohol have been found to have protective qualities against birth defects of

prenatally alcohol exposed babies in South Africa (Viljoen et al. 2001). Furthermore, possible transmission of the epigenetic changes due to alcohol metabolism and effects on sperm can cause changes in expression of critical fetal development genes (Ouko et al. 2009). Alcohol reduces levels and activity of DNA methyltransferase, which induces hypomethylation in the normally hypermethylated DNA in sperm. It is suggested that this may be the reason why the chronic alcohol use in men demethylates sperm DNA in 2 differentially methylated regions (Ouko et al. 2009).

While the exact mechanism of ethanol's teratogenicity is still unknown, many have postulated possible explanations. Alcohol exposure may influence fetal growth in humans by inducing oxidative stress; fetal consumption of nitric oxide subsequently increases, which can cause vasoconstriction and abnormal blood flow (Kay et al. 2006). Indeed, markers for oxidative stress were found in placental tissue two hours after ethanol perfusion (Kay et al. 2006). Another explanation focuses on disruption of endocrine system. Haley et al. (2006) found 5-7 month old infants had higher cortisol levels and heart rates under emotional duress, which implicates disruption of the limbic, hypothalamic pituitary-adrenal axis. Computational studies have reported that ethanol appears to affect the MAPK, TGF-β, and Hedgehog signaling pathways, which are essential for modulating cellular function and embryonic growth (Lombard et al. 2007). Furthermore, neurodegeneration has been documented in many animal models exposed to alcohol, which might be the basis for lighter brain mass of individuals with FAS (Ikonomidou et al. 2000).

To better understand FAS and FASD, animal models, including mice, rats, and zebrafish, have been used and homologous morphological and behavioral phenotypes have been noted (Randall and Taylor 1979; Bilotta et al. 2004; Ninkovic and Bally-Cuif 2006; Summers et al.

2009; Incerti et al. 2010; Akers et al. 2011; McCollum et al. 2011; Ali et al. 2011). Olney et al. (2002) reported that prenatal ethanol exposure resulted in apoptotic neurodegeneration in various parts of the murine brain and suggested that ethanol's properties as both NMDA antagonist and GABAmimetic may causes neurodegeneration. Ikonomidou et al. (2000) further provided that ethanol acts by blocking NMDA receptors and up-regulating activation of GABA receptors to cause widespread neurodegeneration of the rat forebrain. Studies focused on FASD prevention have noted the use of supplemented dietary zinc and choline to protect against the dysmorphology, aberrant behavior, and learning deficits of mice prenatally exposed to ethanol (Thomas et al. 2000, Ziesel 2006, Thomas et al. 2007, Summers et al. 2009). Other studies that concentrate on treatment of FASD, have found that there was some recovery in the learning deficits of mice prenatally exposed to ethanol through the administration of neuroprotective peptides such as D-NAPVSIPQ and D-SALLRSIPA (Incerti et al. 2010).

Logistically, the zebrafish is a much more efficient model to use than its mammalian counterpart. It is cheaper to purchase and maintain zebrafish than mammal models, and handling zebrafish during experiments is easier compared to mammals. The zebrafish has only recently been recognized as a valuable model to study FAS, and the teratogenic effects of embryonic exposure to ethanol have been documented; resulting phenotypes are quite homologous to the phenotypes observed in humans with FASD and experimental rats (Bilotta et al. 2004; Carvan et al. 2004; Rico et al. 2007; Fernandes and Gerlai 2009; Marrs et al. 2010; Sylvain et al. 2010; Ali et al. 2011). In addition, the zebrafish genome is highly conserved, has been sequenced, and is currently being annotated (Chen et al. 1996; Postlethwait et al. 1998; Norton et al. 2010). This makes the zebrafish a great model for studying how ethanol's teratogenic effects relate to certain conserved genes that are involved in development of the embryo. Finally, Ali et al. (2011) and

Levin (2011) identify advances in technology that make the zebrafish an even better tool today to understand various diseases and bridge the gap between in vitro and rodent models.

The zebrafish's development has been widely observed and studied. According to Kimmel et al. (1995), the 72 h development of the zebrafish embryo consists of seven stages – zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching period. The zygote period starts at the 0 h post-fertilization (hpf) and ends at 0.75 hpf; this period represents the first zygotic cell cycle. The cleavage period from 0.75 – 2.25 hpf denotes the second to the seventh metasynchronous cell cycles. During the blastula period from 2.25 hpf -5.25 hpf, the embryo goes through asynchronous cell cycles, begins midblastula transition and epiboly, and creates a border between the yolk cell and the blastodisc. During the gastrula stage from 5.25 to 10 hpf, the germ ring, epiblast, hypoblast, and evacuation zones are visible. In addition, the embryo's brain rudiment thickens and the notochord becomes distinct. Segmentation occurs from 10 hpf to 24 hpf, during which the embryo develops somites, pharyngeal arch, and neuromeres. Muscular twitches and extensions of the tail also arise during segmentation. In the pharyngula period from 24 hpf to 48 hpf, the heartbeat, vascularization and circulation in the yolk, pigmentation, fin folds, touch reflexes, and spontaneous movements occur. During the hatching period from 48-72 hpf, the embryo completes rapid morphogenesis of primary organ systems and cartilage development of the head and pectoral fins. By 72 hpf, the hatchlings are considered larvae; they exhibit food seeking and active avoidance behaviors.

After embryonic exposure to ethanol, zebrafish larvae exhibit morphological abnormalities that are distinct from larval morphology documented under controlled conditions, without exposure to any contaminants. These differences are similar to the phenotypes identified for humans with FASD. Morphological abnormalities in zebrafish are both dose- and stage-

dependent (Bilotta et al. 2004: Dlugos and Rabin 2010), as has been described for humans. The dose-dependent and stage-dependent manners of the teratogenic effects of ethanol indicate that there is a range of severity in morphological abnormalities due to embryonic ethanol exposure. Bilotta et al. (2004) found that zebrafish hatchlings that have been exposed to ethanol exhibit smaller eyes, larger heads and yolks, and malformations in the heart. They noted that the severity of the abnormal phenotype is dependent on exposure and the dose of the ethanol treatment. As concentrations increased from 1.5% to 2.9% (v/v), the severity of the zebrafish's abnormalities and mortality rate increased. Additionally, though there were no overt differences in morphology between the hatchlings exposed to 1.5% ethanol and to the control solutions from 0 to 8 hpf, there were slight physical differences that were detectable only after taking measurements. These two findings suggest that there is a dose-dependent relationship between ethanol exposure and morphological abnormalities. Furthermore, ethanol exposure during the first 24 hours of development affected the zebrafish more than ethanol exposure during any other 24-hour period, which suggests that at least certain morphological abnormalities are stage-dependent or stage-specific.

Another dimension to the range and severity of ethanol's teratogenic effects is that of organ function. Specifically, zebrafish exposed to 1.5% ethanol at an early stage of development (0 to 8 hpf) exhibited morphologically normal hearts but heart rates were significantly slower than the heart rates of the controls (Bilotta et al. 2004). This report demonstrates that there are more subtle changes in the zebrafish's bodily function that are not readily seen. Indeed, Matsui et al. (2006) found that while higher levels (2% v/v) of ethanol exposure inhibit photoreceptor development and cause hypoplasia of the optic nerve, lower levels (1% v/v) of ethanol exposure still affect the photoreceptor function without causing any changes in retinal morphology. To

better understand the teratogenic effects of embryonic ethanol exposure, both the morphology and the functionality of various organs of the zebrafish should be monitored.

In the past few years, there has been a bourgeoning interest in the behavior of zebrafish. Norton et al. (2010) found that adult zebrafish are great models for studying complex behavior; conserved regulatory processes that are present in both zebrafish and mammals regulate learning, memory, aggression, anxiety, and sleep. The realization that the behavior of the zebrafish is regulated by conserved genes is important because research today focuses on the isolation and molecular analysis of zebrafish behavior mutants to allow annotation of the novel behavioral control genes. By manipulating various parts of the zebrafish genome or by targeting various aspects of the development of the zebrafish, researchers can link behaviors and phenotypes to the various genes in the zebrafish.

Before we can definitively understand the zebrafish genome in relationship to its behavior, there must be a general understanding of the zebrafish behavior. Gerlai et al (2000) performed four tests that observed four behavioral phenotypes - aggression, group preference, antipredation, and light preference- of adult zebrafish after chronic exposure to ethanol in their environment. They found that the zebrafish exhibited greater preference to remain in the lower third of the tank as environmental ethanol concentration increased. Interestingly, low alcohol concentrations (0.25% v/v) increased aggressive behavior responses elicited from the competitive aggression test. The fishes' preference to shoal was directly and negatively related to the dose of the treatments. Low alcohol concentrations again increased antipredator behavior, but this behavior dropped at higher concentrations of ethanol exposure (1% v/v). Finally, zebrafish exposed to higher concentrations of ethanol continued to avoid the dark compartment of the tank while the control and lower-dosed zebrafish readily habituated to the dark compartment.

In this set of experiments, I tested the hypothesis that chronic and acute exposures to low concentrations of ethanol applied at different times during the early developmental period have important and significant effects on the morphology, physiology, and behavior of hatchling zebrafish. I assigned embryonic zebrafish to six chronic ethanol treatments and five acute ethanol treatments. These treatment groups were designed to target specific periods of development of the zebrafish embryo. In each treatment group, the embryos were exposed to tank water (control), 0.5% ethanol (v/v), or 1% ethanol (v/v) solutions. I then used digital images to measure heart rates and eye and yolk widths. I compared values of these measurements among fish within each treatment group to assess the effects of ethanol exposure on the morphology of the embryos. To gauge the ethanol concentration within the embryos themselves, I completed assays using yeast alcohol dehydrogenase according to methods by Reimers et al. (2004). Three months after fertilization, the now adult zebrafish were subjected to a light preference behavioral test.

The chronic and acute treatments provide for an important comparative experimental design. First, I could compare the teratogenic effects of low dose chronic ethanol exposure in my experiment to those reported for previous experiments that use high doses of ethanol during the same exposure time period. For example, Bilotta et al. (2004) demonstrated the teratogenic effects on the morphology of the zebrafish after 1.5% ethanol (v/v) exposure throughout periods of the zebrafish development. I predicted that these same morphological changes could occur after lower doses of ethanol exposure (0.5% and 1% v/v). Second, while there are many studies published on the effects of chronic exposure on the morphology and function of various zebrafish organs, there is little empirical knowledge on the effects of acute exposure on development. Exposing the zebrafish embryos to ethanol acutely may provide results that contribute to a better understanding of ethanol effects on zebrafish development. Finally, by

increasing the frequency of acute exposure, the zebrafish is exposed to ethanol for short periods of time at various points of its development. This may better represent the kind of alcohol exposure a human fetus could experience. Increasing the frequency of acute exposure may increase ethanol's effect on zebrafish development because the embryo will be exposed to ethanol at multiple stages of it development.

There is very little known about the effects of low concentrations of ethanol exposure on embryonic development (Fernandes and Gerlai, 2010) so the low dose treatments in my experiment are also valuable. While it is established that high doses of ethanol have strong teratogenic effects on the zebrafish, it is important, and perhaps more relevant, to study the effects of low doses of ethanol on development in the context of human disease. Because fetal alcohol syndrome is a spectrum disorder and because zebrafish exposed to ethanol exhibit homologous phenotypic characteristics, it is important to investigate if a similar spectrum of morphological traits exist in zebrafish at low doses of ethanol. Studies on the effects of low doses of ethanol embryonic exposure in zebrafish can aid our understanding of the entire spectrum of FASD.

This experiment focused on the ratio of eye width and yolk width as an indicator of morphological effects of ethanol. Eye size is an important indicator of prenatal or embryonic ethanol exposure in both humans and zebrafish models. Likewise, Bilotta et al. (2004) noted yolk sac edema after embryonic ethanol exposure, and this symptom has been reported for other studies. Because smaller eyes and larger yolk sacs are classic teratogenic effects of ethanol on the morphology of the zebrafish (Bilotta et al. 2002, Bilotta et al. 2004), a smaller eye width:yolk width may be used to pronounce teratogenic effects. Larger ratios reflect larger eye width or the smaller yolk width, which are both morphological characteristics of unexposed larval zebrafish

exhibiting normal development. I anticipated ratios to decrease in a dose dependent manner and in a stage dependent manner.

To gauge the effect of ethanol on organ function, I recorded the heart rate of the hatchlings. The first evidence of heart formation occurs at 5.5 hpf (Fishman and Chien 1997) when precardiac cells migrate from the epiblast. About 8 h later, the myocardial plate is evident, and 6 h afterwards, a single heart tube is generated. By 22 hpf, this tubular heart contracts and the first heartbeats are formed. At 33 hpf and 48 hpf, the tubular heart loops and forms cushions. Additionally, there are eight genes intimately involved in heart morphology and 14 genes in the heart beat of the zebrafish (Chen et al. 1996). The development of the heart and start of the heart rate is a genetically intimate and lengthy process. By observing the effects of low doses of ethanol at various times in the zebrafish's development, I anticipated seeing dose and stage dependent effects on the heart rate of the zebrafish.

Knowing the change in behavior after embryonic ethanol exposure is crucial for many reasons such as further exploring the use of zebrafish in studying the human disorder and understanding how ethanol exposure can change the basic and conserved behaviors of vertebrates. While Fernandes et al. (2010) demonstrated low shoaling activity in zebrafish that were embryonically exposed to ethanol, there have not been any other behavioral tests performed on embryonically-exposed zebrafish. Zebrafish are highly dependent on their eyesight to forage food, view conspecifics, and detect predators. While all zebrafish thus depend on a lit environment to view their surroundings, after some time without any stimuli, zebrafish learn to habituate and explore darkened portions of a tank (Gerlai et al. 2000). By observing the effects of low doses of ethanol during development on the light/dark preference of the zebrafish, I

anticipate seeing zebrafish, whose eyes are likely to be deteriorated, to prefer the lit portion of the tank and not explore the darkened portions of the tank.

Materials and Methods

General Procedures

I used AB strain zebrafish from a breeding colony maintained in the Biology Department at Colby College and University of Maine at Orono. As illustrated in the flow diagram in Figure 1, females and males were mated, and the resulting embryos were exposed to ethanol according to assigned treatment groups. I took digital images of the hatchlings 72 hours post fertilization. . All hatchlings were placed in small tanks equipped with filters and maintained at 25-27 ºC until hatchlings were three months post hatching. I subjected these mature fish to behavioral tests. (Figure 1). I completed ethanol determination assays to estimate the ethanol concentration present in the embryos after exposure.

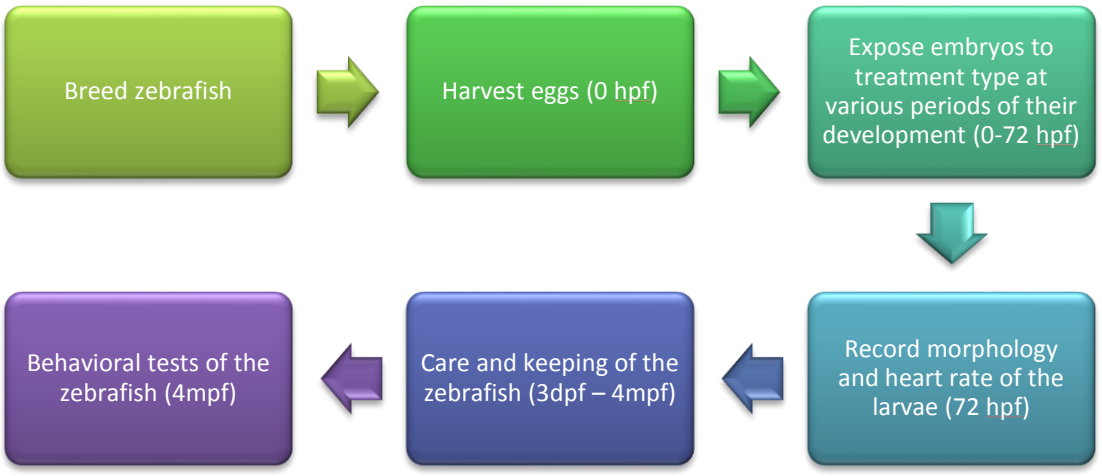

Figure 1. General procedure of the experiment.

Zebrafish housing and care

Retired AB strain breeding zebrafish (*Danio rerio)* were donated by the Colby College Biology Department and by the University of Maine Biology Department. I separated the fish by sex and housed the males and females in two clear 15 gal tanks equipped with filters and heaters set at 27ºC. Tank water was distilled water enriched with Coralife Scientific Grade Marine Salt (5 g/20 L of distilled water). Breeder fish were fed twice a day five days a week with Hikari Tropical Micro-Pellets and fed brine shrimp (INVE Aqualculture Nutrition: Artemia Cysts) three times a week. Hatchling zebrafish were housed in clear 3 gal containers equipped with filters and heaters set at 25-27 ºC. They were fed Zeigler Shrimp Larva Diet twice daily. Pellet size shifted as hatchlings grew and included 100 um, 250 um, or 400 um pellets. Tank water was changed every week or as needed. The lighting cycle was set to a 14-hour day and 10-hour night cycle.

Breeding Protocol

To obtain eggs, I placed adult female and male fish together at a ratio of 5:7 or 5:8 in breeding tanks specifically designed for easy egg harvesting. The breeding tanks were filled with tank water and heaters set to maintain the water temperature at 25-27 ºC. Any eggs produced were assumed to be fertilized, collected at noon, and exposed to ethanol according to their designated treatment group during their development.

Treatment Groups

I categorized treatments by exposure time and by ethanol treatment. Exposure times were either chronic or acute. Because the female breeder zebrafish deposit eggs in the morning, between 0800 and 1200, I designated 1200 as the 0^{th} hour of fertilization. Embryos designated to the chronic exposure were exposed to the ethanol solutions at 0-8, 0-10, 0-24, 10-24, 24-48, and

48-72 hours post fertilization (hpf). Thus, the embryos were exposed for 8, 10, 14, or 24 hours starting immediately to several hours or days post fertilization (Figure 2).

Embryos assigned to the acute group treatments were exposed to the ethanol solutions for shorter periods of time. Treatment groups varied in the length of exposure time and in the number of times the embryos were exposed to the treatment. Embryos in one acute group (from here on called 1h x 4) were exposed for 1 h at four different times during the development period 0, 24, 48, and 71 hpf. Embryos in a second acute group (4 h x 1) were exposed to alcohol for 4 h once during the development period starting at 0 hpf. Embryos assigned to the third acute group (4 h x 2) were exposed to alcohol for 4 h twice during the development period starting at 0 and 24 hpf. Embryos in the fourth acute group (4 h x 3) were exposed to alcohol for 4 h three times during the gestation period starting at 0, 24, and 48 hpf. Embryos in the fifth acute group (4 h x 4) were exposed to ethanol for 4 h four times during the gestation period starting at 0, 24, 48, and 68 hpf. Within each chronic and acute treatment group, embryos were further sub-divided and assigned to ethanol concentration treatments of 0% (control), 0.5%, or 1% ethanol (v/v).

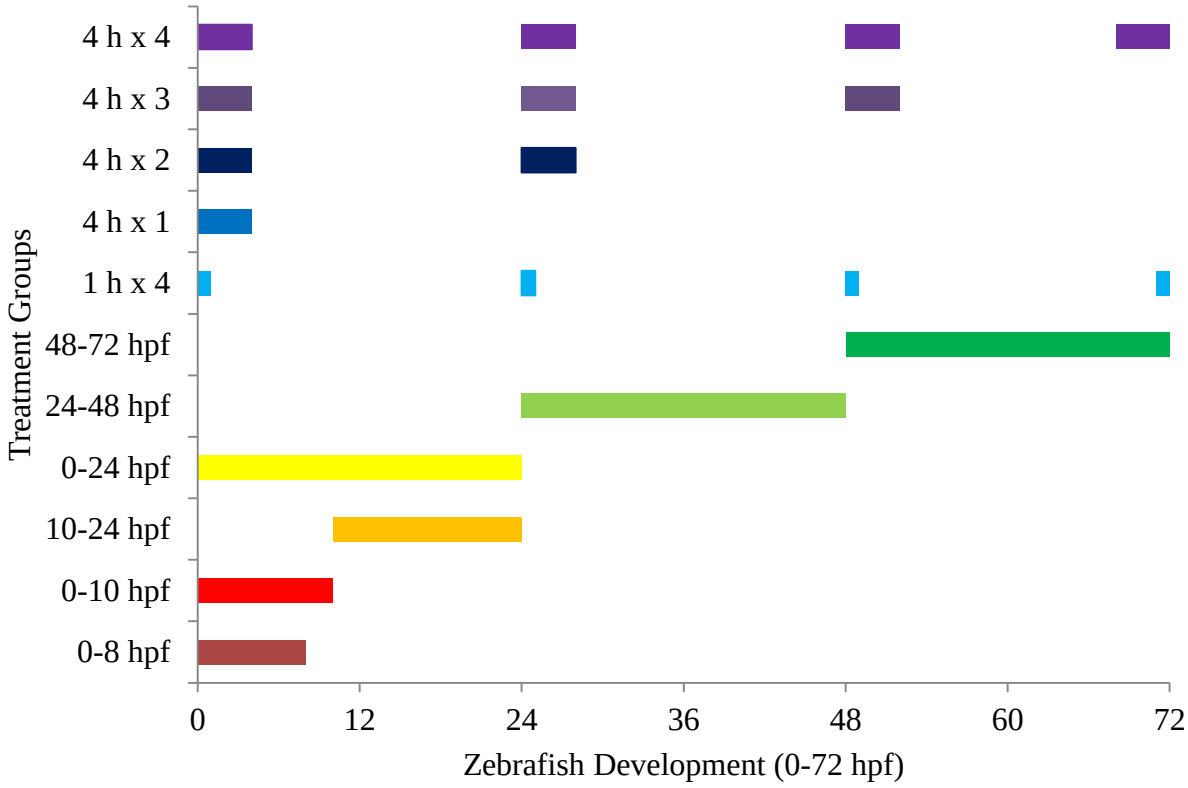

Figure 2. Timeline of the chronic and acute treatment groups along the development of the zebrafish.

Treatment Protocol

I used a consistent protocol for all treatments of zebrafish embryos, starting with an initial rinse of diluted Zep Perosan ® solution to sanitize the embryos and control against any infections. Embryos were then divided and placed in 2-oz glass jars identified by treatment group. The jars contained 20 mL of tank water, or a treatment solution during exposure periods. After the exposure time, the liquid was siphoned using a micropipetter and embryos strained from the jar using a cup with a 10-um strainer at the bottom. The embryos were then rinsed with 10 mL of deionized water and then placed back in the jar with tank water and secured lid. The jars were placed together in 5-gal aquaria equipped with heaters to maintain water at 25-27ºC. Debris in

the jars was not removed unless the embryos underwent their treatment regime. Control embryos underwent the same procedure to account for any handling effects of the eggs. At 72 hours post fertilization, I obtained digital images of the hatchlings' torsos and heads and recorded their heart rates. Hatchlings were afterwards placed in housing as described above.

Ethanol Determination Assays

I estimated the amount of ethanol present within the embryos using an ethanol determination assay developed by Reimers et al. (2004). I used yeast alcohol dehydrogenase and NAD+ to convert ethanol to acetaldehyde. I used a Hitachi U-3010 spectrophotometer to monitor the accumulation of the acetaldehyde through a colorimetric test. I mixed ethanol standards from 0% to 1% at .1% increments and developed a standard curve for alcohol dehydrogenase activity after 10 minutes of incubation.

When determining the ethanol concentration in the embryos, I first completed the treatment protocol and exposed the eggs with ethanol. Immediately after the exposure, instead of rinsing the embryos with deionized water, I homogenized the sets of 30 embryos according to Reimers et al. (2004), then extracted the supernatant from the embryos and used them in the assay.

Statistical Analysis of the Morphology and Heart Rate

To determine differences in morphology and heart rates of hatchlings in different treatments, I used the Kruskal-Wallis test to first determine if there were treatment effects among the comparison groups, then the Mann-Whitney U-test to complete pair-wise comparisons. I completed these tests on STATA11, and the p-values are reported for any significant differences identified through an experiment-wise alpha p value of 0.017. I compared the ratio of eye width

to yolk width and the heart rates within each treatment group and across treatment groups. These tests were used because of the low sample size in the treatment groups. The eye width: yolk width was used because the ratio combines two elements, eye width and yolk width, which are affected by embryonic ethanol exposure. This ratio will amplify any morphological changes due to the exposure to ethanol, which may help to compensate for small sample sizes. The heart rate was measured to gauge teratogenic effects on organs.

Behavior Tests- General Procedures

At three months of age post hatching, the mature zebrafish were subjected to four behavioral tests. To test for light preference, each zebrafish was placed in a 3 gal tank by itself and tests were conducted sequentially. The zebrafish had 2 min to recover between tests and had 5 min to acclimate to any new stimulus before behavioral observations were recorded. Each fish was observed and behaviors recorded for 10 min. All trials were recorded with a Sony DCR-SR100 camcorder.

Behavioral tests were conducted using two tanks (Figure 2). A 15-gal tank, referred to as Test Tank, or TT, housed either conspecifics used in the shoaling test or the predator model used in the anti-predator response test. A 3-gal tank referred to as Experimental Tank, or ET, held the focal zebrafish. The two tanks, TT on the left and ET on the right, were visually isolated using a piece of cardboard at the start of and end of the shoaling and anti-predator response trials. Both tanks were covered on three sides by cardboard, and the fourth was used to make observations and records. The tanks both held tank water, and the height of the tank water was the same in TT and ET. TT was divided into quadrants by drawing three vertical lines directly on the tank. Three vertical lines and one horizontal line were also drawn on ET to create eight equal sections. To

prevent biases, a student researcher randomly selected the focal zebrafish without my knowledge. The identity of the focal zebrafish was revealed only after analysis of the behavior videos.

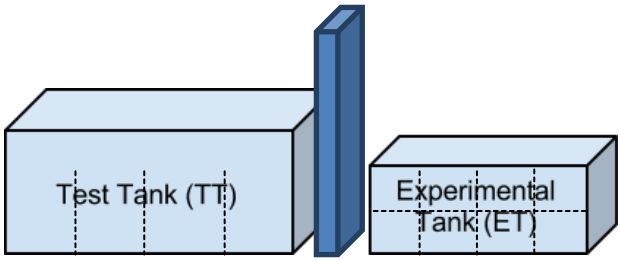

Figure 2. Schematic of arenas used for the behavioral experiments. An opaque barrier prevents visual contact between fish in the Test Tank (TT) and Experimental Tank (ET) (not to scale). The dotted lines represent the lines were drawn to divide the tanks into quadrants.

Light/Dark Test

After 2 min of recovery, a three sided box was placed on the right half of ET, which illuminated the left half of ET and darkened the right half of ET. There were no barriers within the tank itself, so the focal zebrafish was free to swim in either the lit or the darkened areas of the tank. Following the first 5 min of habituation, I recorded the period of time the focal zebrafish was observed swimming in the fully lighted portion and the amount of time it spent in the covered portion of the tank. Video recordings from each trial were analyzed using JWatcher 1.0 © (Blumstein et al. 2001) to quantitatively analyze zebrafish behavior and the percent time the fish spent in the left four sections and in the dark was calculated.

Statistical Analysis of the Behavior

To analyze the morphology of the hatchlings, I used the Mann-Whitney U and Kruskal - Wallis tests on STATA11 to analyze the percent time the focal zebrafish occupied the uncovered

and the covered halves of the tank. These tests were used because of the low sample size in the treatment groups. All significant differences had a p-value of less than 0.05.

Results

Using the experimentally determined standard curve, the ethanol concentration inside the embryos in the 0-24 hpf treatment group was on par with the exogenous ethanol solution concentration (Figure 4).

Chronic Treatment

Morphology

The average eye width:yolk width of hatchlings exposed to the 1% ethanol solution was significantly lower than that of the control hatchlings in the 10-24 hpf group ($p < 0.001$, Figure 5). In the 48-72 hpf group, the ratio of hatchlings exposed to 0.5% ethanol was significantly higher than the ratios of hatchlings exposed to either the control or 1% treatments ($p = 0.012$). Average ratios were varied in the rest of the chronic groups for the hatchlings exposed to the other treatments, and the differences were not significant.

Heart Rate

The heart rates of hatchlings from the 0.5% and 1% treatments in the 0-24 hpf chronic group was significantly faster than the heart rate of control hatchlings in each group ($p < 0.017$, Figure 6). In the 24-48 hpf group, there were significant differences among the heart rates of hatchlings from all treatment groups ($p < 0.01$). The heart rate of hatchlings exposed to 1% was the fastest, and heart rate of hatchlings exposed to 0.5% was the slowest. In the 48-72 hpf group, the heart rate of hatchlings exposed to 1% ethanol solution was significantly faster than the heart

rates of hatchlings exposed to the control and to 0.5% ethanol solutions ($p < 0.001$). Heart rates for the rest of the groups were not quite significantly different under the experimental-alpha p-value ($p < 0.03$)

Acute Treatment

Morphology

The average eye width:yolk width of the hatchlings exposed to 1% ethanol in the 4 h x 4 group was significantly smaller than the ratios of the control hatchlings in their respective groups ($p= 0.007$; $p = 0.034$, Figure 7). There was no significant difference in the ratios among hatchlings of the treatments within 4 h x 3, 4 h x 2, and 4 h x 1 groups, though 4 h x 2 was close to significant ($p = 0.03$).

Heart Rate

There were significant differences in the heart rates of zebrafish in all acute treatment groups except 4 h x 3 and 4 h x 1. In the 1 h x 4 group, the heart rate of hatchlings treated with 1% ethanol solution was significantly faster than the heart rates of the hatchlings in the control and 0.5% ethanol treatment ($p < 0.001$, Figure 8). The heart rates of hatchlings exposed to 0.5% and 1% ethanol were also significantly faster than the heart rate of control hatchlings in the 4 h x 4 groups ($p = 0.0008$, $p = 0.0001$). 4 h x 3 was close to significance ($p < 0.03$).

Behavioral Test

Though behavior tests were conducted on many of the chronically exposed zebrafish, only the data for zebrafish of the 10-24 hpf group were analyzed because of the amount of available data. Focal zebrafish exposed to 0.5% ethanol from 10-24 hpf spent significantly more

time in the lit portion of the experimental tank than the control fish ($p < 0.05$, Figure 9). The sample size for the behavior of zebrafish exposed to 1% ethanol from 10-24 hpf was too small for further analysis. Due to the timescale of this thesis, further replication of the experiment could not be conducted.

Discussion

The results of this experiment provide evidence that specific-stages of development are more sensitive to embryonic exposures of low doses of ethanol resulting in the morphology of the zebrafish, that the heart function is affected in a dose-dependent manner, and that the morphologically affected chronic treatment group (10-24 hpf) demonstrated changes in light/dark preference. When the zebrafish embryos were exposed in a chronic manner, the morphology of the zebrafish exposed to the 10-24 hpf treatment group reflected a dose dependent change while the morphology of those exposed in the 48- 72-hpf group did not follow the same pattern. Heart rates were faster for almost all larvae in the chronic treatment groups with significant differences in the larvae exposed for 24 h. This indicates that long exposures to low doses of ethanol affect the physiology of the zebrafish without causing measurable changes in morphology. When zebrafish embryos were exposed in an acute manner, almost all treatment groups exhibited the same pattern on the eye and yolk size, but significant differences were only apparent in the treatment groups with four exposure times. The changes in the heart rate again demonstrate that physiological changes can occur without measurable morphological changes. The heart rates of embryos in the 4 h x 3 and 4 h x 1 treatment were not statistically different; however, the consistent heart rate demonstrated in all treated and untreated larvae in 4 h x 1 suggests a possible threshold at which low doses of ethanol at short durations or specific stages of development may not have physiological and morphological effects on the zebrafish.

Preliminary behavioral data for the 10-24 hpf treatment group demonstrates exposed zebrafish did not readily habituate to the dark areas of the tank.

Morphology

The period of most teratogenicity appears to occur with ethanol exposure during 10-24 hpf of the zebrafish development. This period of development is the segmentation stage. From Kimmel et al. (1995), Iknow that the segmentation stage is important for somite formation, neuromere formation, extension of the yolk, otolith formation, tail budding, and initiation of organogenesis. The segmentation phase of the zebrafish's development may be more sensitive to ethanol than other phases in terms of eye width and yolk size. These morphology results corroborate existing reports on embryonic ethanol exposure. Ali et al. (2011) performed an experiment using 10% ethanol (v/v) for 1 h exposure times at various points of the zebrafish's development. They found that expression of *prim-6* and *prim-16*, which occurs during the pharyngula phase, are critical times for ethanol sensitivity. While results from my study suggest that the segmentation phase was most sensitive, it is important to keep in mind that Iassumed that the 0^{th} hour of fertilization was at 1200. If the eggs were fertilized before 1200, which is most likely to case since the eggs were treated at that time, our results line with Ali et al.'s (2011) findings.

Interestingly, only the ratio of the 10-24 hpf group and three of the acute groups decreased sequentially as the dose of ethanol increases. Other treatment groups displayed different patterns. In the other chronic treatment groups, the ratio increased in the hatchlings exposed to 0.5% ethanol in nearly all other treatment. The 48-72 hpf group significantly demonstrates this pattern. I expected the 4h x3 treatment group to demonstrate similar behavior as the 4h x 2 and 4h x 4 groups, but instead, the ratio of the 1% treated hatchlings was larger than

the ratio of the control. These inconsistencies may be a result of experimental flaw, as may be the case for the 4h x 3 group, or may hint to an unique effect of chronic exposure to lower doses of ethanol.

Heart Rate

Unlike the morphology of the zebrafish, the function of the heart seems less sensitive to the low doses of ethanol exposure. Among the chronic groups, only the larvae exposed for 24 h demonstrated significant increased heart rate. The similarity in the heart rates of larvae exposed to 0.5% and 1% in the 0-24 hpf treatment group support the Bilotta et al. (2004) finding that there may be higher sensitivity to ethanol during the first 24 h of development. Because the rest of the heart rates in the chronic groups were close to significance, further analyses should expand the sample size.

Because the development of the heart is a lengthy process with many crucial and most likely interacting genes, the sensitivity of the hatchlings to the low concentrations of ethanol seen in the results is logical. Further research should explore how the mechanism for ethanol's teratogenicity influences the genes found most important to heart generation and whether the heart rate of the exposed zebrafish remains compromised throughout its lifetime.

There have not been any previous studies that resemble the acute treatment protocol in this experiment. The insignificant changes in the morphology and heart rate of the 4 h x1 suggest a possible threshold under which the embryo can develop normally despite exposure to ethanol. Additionally, the acute results are a novel demonstration of how at similar exposure frequencies, longer durations exposure may amplify the teratogenicity of low doses of ethanol that is not seen in shorter durations of exposure. For example, while the 0.5% ethanol exposed hatchlings in the 1 h x4 group are relatively equal to the heart rate of the control, the 0.5% ethanol in the 4 h x4

group have heart rates as fast as the 1% exposed hatchlings. Along with data from 4 h x1, the comparison between 1 h x4 and 4 h x4 may possibly demonstrate the ability of the embryo to resist the effects short exposures times of low doses of ethanol.

When comparing the effects of ethanol exposure on the heart rate of hatchlings that were exposed at various frequencies for 4 h, the results varied slightly from what might be expected. I had expected to see the heart rate of the larvae exposed to 0.5% ethanol become increasingly aberrant as the frequency increased because ethanol would affect various points during the formation of the heart, which occurs throughout development. In other words, increases in dose with constant frequency or increases in frequency with constant dose may increase the likelihood of causing physiological changes in the zebrafish. The heart rate in larvae exposed just once to 0.5% ethanol was not different from that of control embryos, but was greater than that of control embryos for the 1% ethanol treated hatchlings exposed three times. However, the heart rates of hatchlings exposed twice decreased with increased ethanol concentration, which seems contradictory when in context with the other acute treatment groups. It may be related to a similar deviation seen in the heart rates of larvae from the 24-48 hpf treatment group. Because there is a four hour overlap (24-28 hpf) between the 4 h x2 and 24-48 hpf treatment groups, future research should investigate the exact morphogenetic events within those times that will cause the heart rate to slow down after ethanol exposure rather than speed up, as is the case in the rest of the exposure time.

Even though the majority of my data on the heart rate of the zebrafish do not align with corresponding heart rate literature, the anomaly found in the 4 h x 2 and 24-48 hpf treatment groups, aligns with the previous studies. Bilotta et al. (2004) and Dlugos et al. (2010) found a decrease in heart rate in zebrafish exposed to 1.5% or 0.5% ethanol in a chronic manner. My

results showed an increase in heart rate upon 0.5% and 1% ethanol exposure except for the 4 h x 2 treatment group. Because most of these data did not correspond with reports from other studies, future studies should investigate the reason for the differences in results. However, even though our results were different than the established literature, because there was a strong pattern in the results of this experiment, the expansion of this experiment will help confirm these results. Further analysis on the zebrafish should be conducted to understand how higher heart rates affect the physiology of the fish.

These results have larger implications on our understanding of FASD because they contribute to the growing literature on the teratogenicity of ethanol on the development of the zebrafish. The findings in this experiment suggest that specific stages of the development of the zebrafish may be more sensitive to the teratogenicity of ethanol compared to other stages. Understanding which stages are more sensitive to the detrimental effects of ethanol has great significance in the understanding FASD and potential therapeutics to help recover a fetus from the effects of ethanol.

Future studies should continue to explore ethanol's dose and stage-dependent teratogenicity, but also include observations on the mechanisms of its teratogenicity. By combining the established literature on the morphogenetic events in the zebrafish development with studies such as this that identify specific stages of development that are most sensitive to the effects of ethanol, future experiments may be able to understand the cellular and biochemical mechanisms behind its teratogenicity. While the exact cellular, biochemical, and genetic mechanisms for ethanol's teratogenicity remain unknown, one suggested mechanism is that ethanol contributes to apoptosis. Carvan et al. (2004) hypothesized that cell death in the central nervous systems influenced the learning and memory deficiency in zebrafish exposed

embryonically to ethanol. Ikonomidou et al. (1999) also point to cell death in the CNS as a potential and molecular pathway for ethanol teratogenicity; though not fully understood, several experiments show reduced brain mass and neurobehavioral issues of individuals with FAS.

Furthermore, future studies on the function of eye function should use specific tests that test the function of various organs. For example, electroretinography (ERG) should be conducted on the zebrafish at the larval stage to understand how morphology of the zebrafish relate the function of their eyes. ERGs can further be used to understand the results of our behavior test, which relies heavily on the eye function of the zebrafish.

Behavior

Understanding the effects of behavior will address one of the core issues of embryonic ethanol exposure. Fernandes et al. (2010) demonstrated disturbances in shoaling activity in zebrafish embryonically exposed to ethanol due to impaired dopaminergic and serotoninergic systems. These behavioral studies could further shed light on the mechanism of ethanol's teratogenicity.

Swimming preference for lit portions of an environment in zebrafish is suggested to occur because zebrafish rely heavily on their eyesight for foraging food, viewing conspecifics, and detecting predators (Gerlai et al. 2000). However, over time, developmentally normally zebrafish have been found to habituate to dark portions of a tank over time without the presence of food, conspecifics, predators, or other stimuli (Gerlai et al. 2000). Data from this experiment point to increased preference for the lit portion of the tank rather than the dark portion of the tank for zebrafish exposed to 0.5% from 10-24 hpf during development, which corresponds well with Gerlai et al. (2000); developmentally normal adult zebrafish exposed to 0.5% and 1% ethanol solutions preferred the lit portion of the tank. The similarity in results of embryonically exposed

zebrafish and zebrafish exposed to ethanol only during adulthood may shed light on the mechanism of ethanol's toxicity in the function of the central nervous system of the zebrafish. To further extrapolate on the similarities between embryonically exposed zebrafish and treated adult zebrafish, further tests should analyze how embryonically exposed zebrafish perform in the same tests that adult zebrafish have been subject to after ethanol exposure.

Furthermore, zebrafish exposed to 10-24 hpf were the only fish used for the behavior experiments. These were the same zebrafish that demonstrated significant changes in morphology when exposed to 1% ethanol but not when exposed to 0.5% ethanol. Because eyesight is important in behavior of light or dark preference, future studies should conduct electroretinograms (ERGs) on the zebrafish to understand how the significant and insignificant morphological changes of the zebrafish affects the function of the eyes, and how the affected eye function relates to the behavior of the zebrafish. The same procedure should also be conducted on zebrafish of other treatment groups that did not demonstrate significant morphological differences.

Conclusion

Results from both chronic and acute treatment groups support the dose dependent and stage specific nature of ethanol's teratogenicity on the development of the zebrafish while results from the behavioral test on the 10-24 hpf treatment groups demonstrated higher preference to lit environments. While results on chronically exposed embryos augment previous reports on the stage specific effects of ethanol, the acute data demonstrate generally the teratogenicity of low dose ethanol as frequency of short exposures increase and behavioral data expands the new literature base on behavior after embryonic exposure. The morphological and heart rate data also provide interesting patterns that raise more questions about the nature of this teratogen. While

the heart rates of both acute and chronic treatment groups demonstrated consistency, morphology data for both treatment groups were not as consistent. The results still implicated that physiological changes can occur despite no noticeable or measurable differences in morphology; this reflects conclusions made in previous work with zebrafish as well as epidemiological studies on individuals with FASD (Bilotta et al. 2004, Matsui et al. 2006, Fryer et al. 2007). Behavioral results of the treated zebrafish and the untreated zebrafish of the 10-24 hpf treatment group demonstrate that treated zebrafish do not habituate to environments, which may suggest preference for lit areas due to changes in eye function. Larger sample sizes, which was a major limiting factor in the accuracy and reliability this experiment's data analysis, are needed in future studies. This will help determine whether or not data that were close to significance are indeed significant. Future studies with larger sample sizes should explore the stage dependent teratogenicity of low doses of ethanol by using both short and long term embryonic exposure within the 10-24 hpf segmentation period and the relationship between eye morphology, function, and zebrafish behavior.

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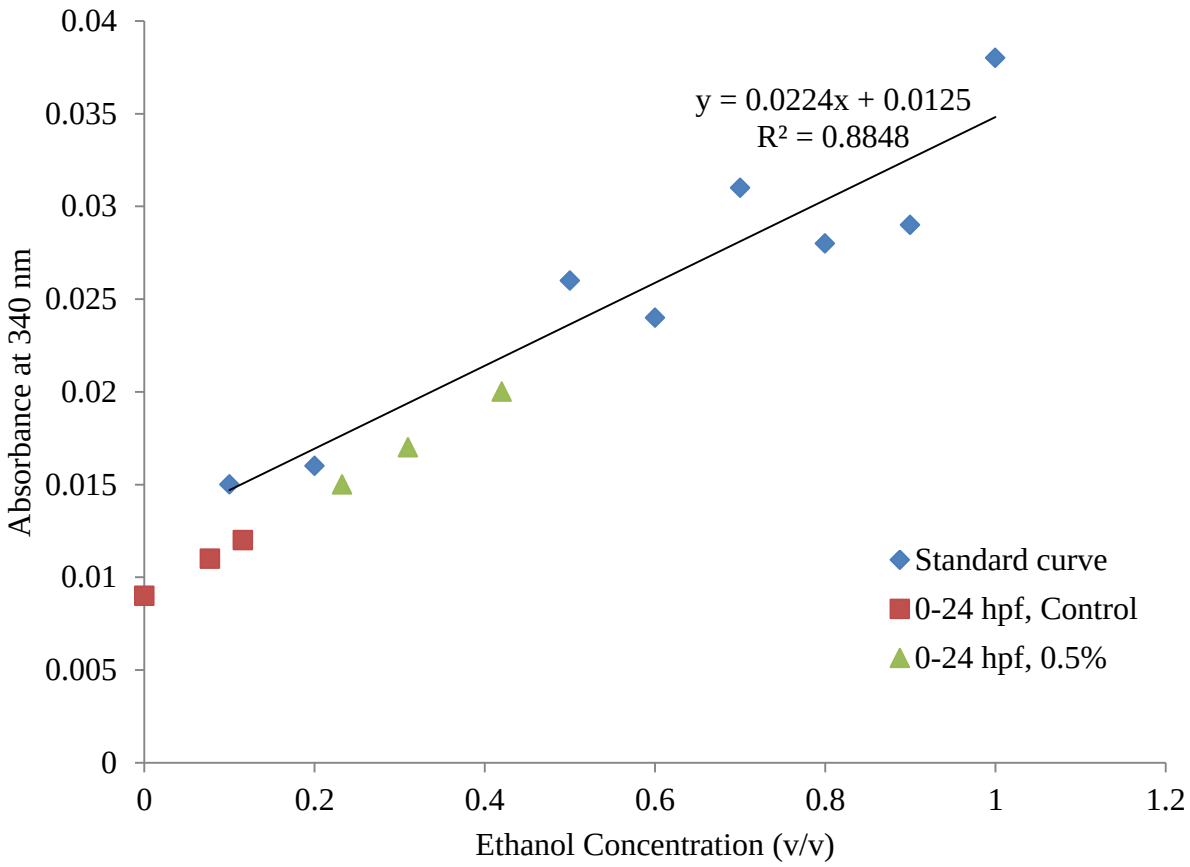

Figure 4. Ethanol determination standard curve and determined ethanol concentration within the control embryos and embryos exposed to 0.5% ethanol in the 0-24 hpf treatment group.

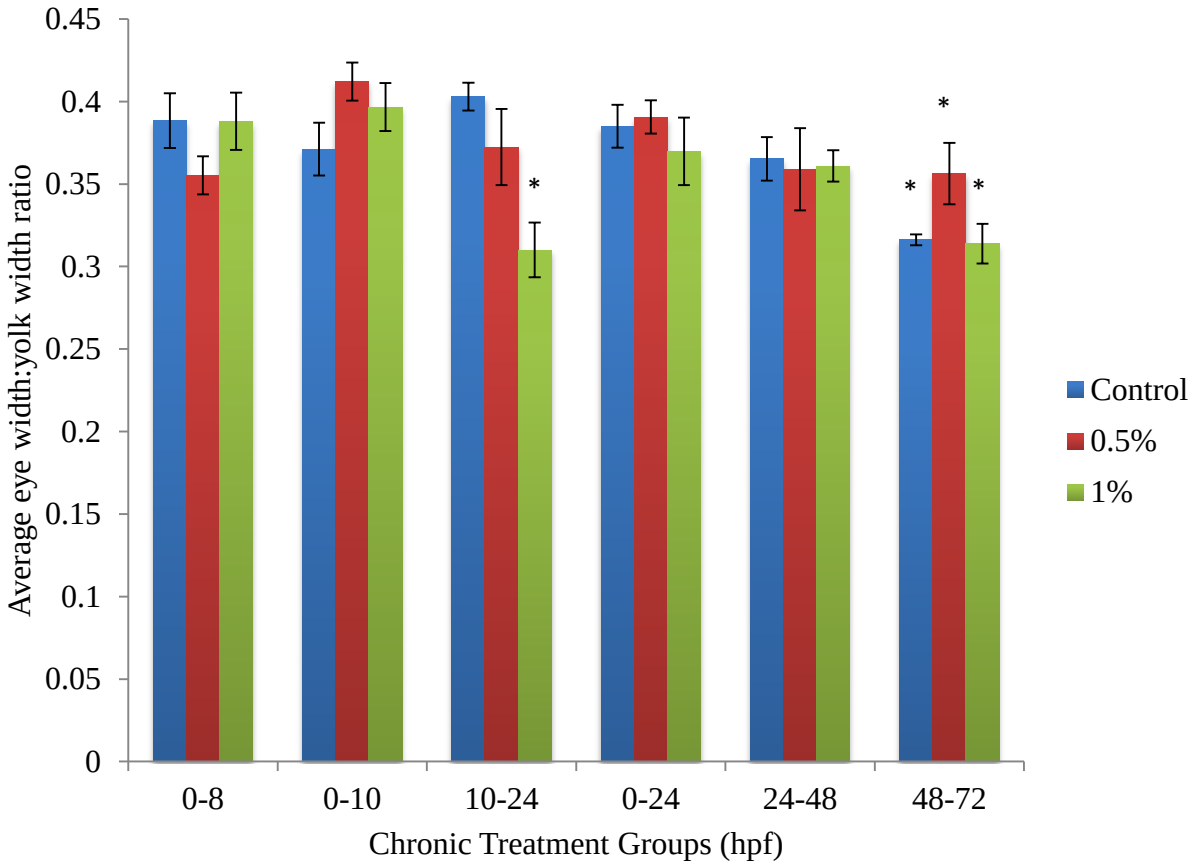

Figure 5. Average eye width to yolk width ratio of hatchlings in six chronic treatment groups (bars reflect +/- 1SE).

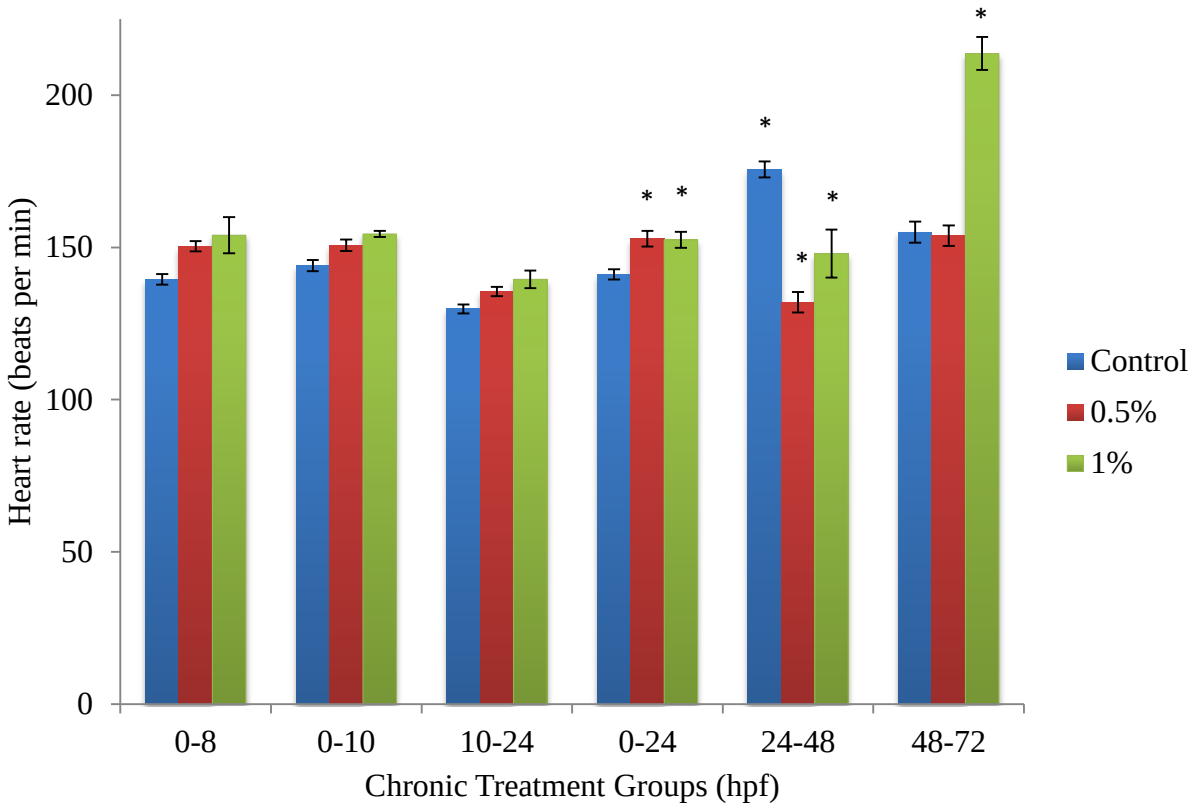

Figure 6. Average heart rates (beats per min) of hatchlings in six chronic treatment groups (bars reflect +/- 1SE).

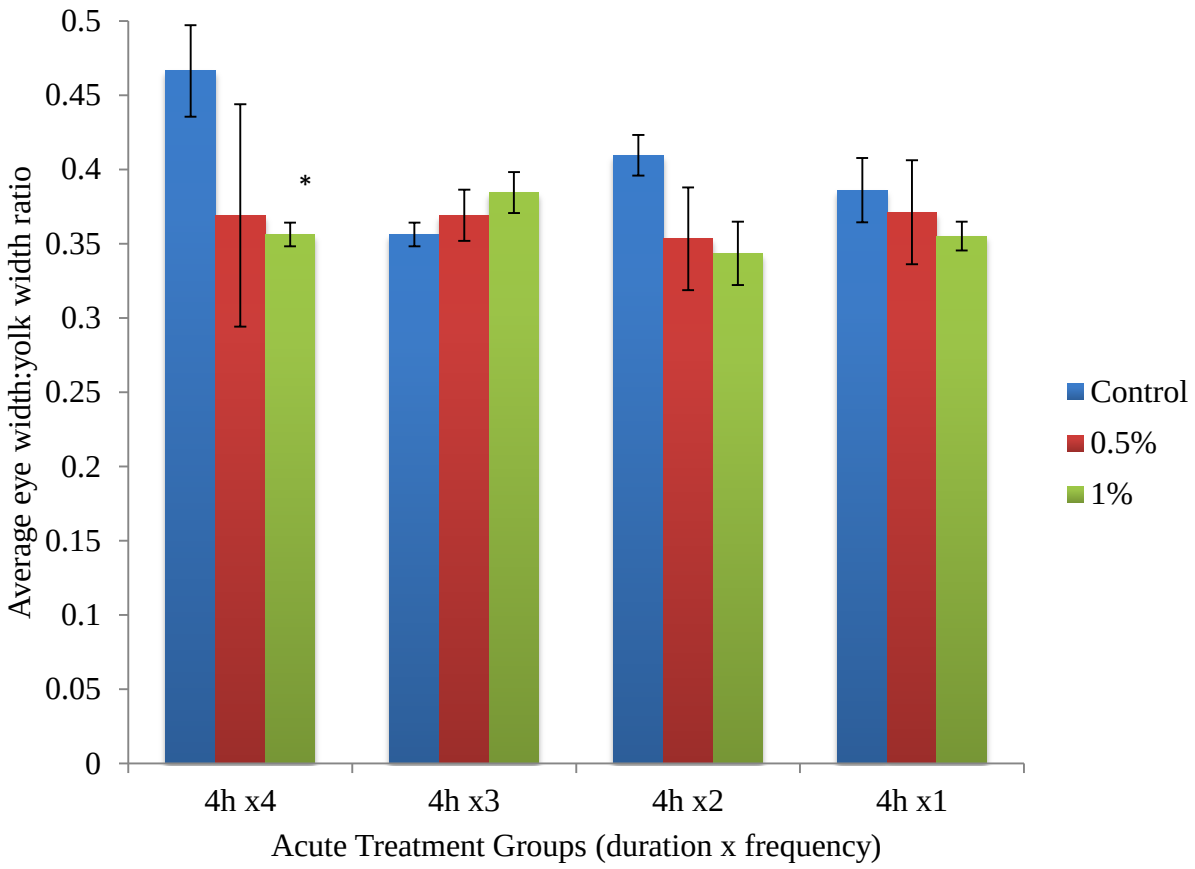

Figure 7. Average eye width to yolk width ratio of hatchlings (72 hpf) of four acute treatment groups (bars reflect +/- 1SE).

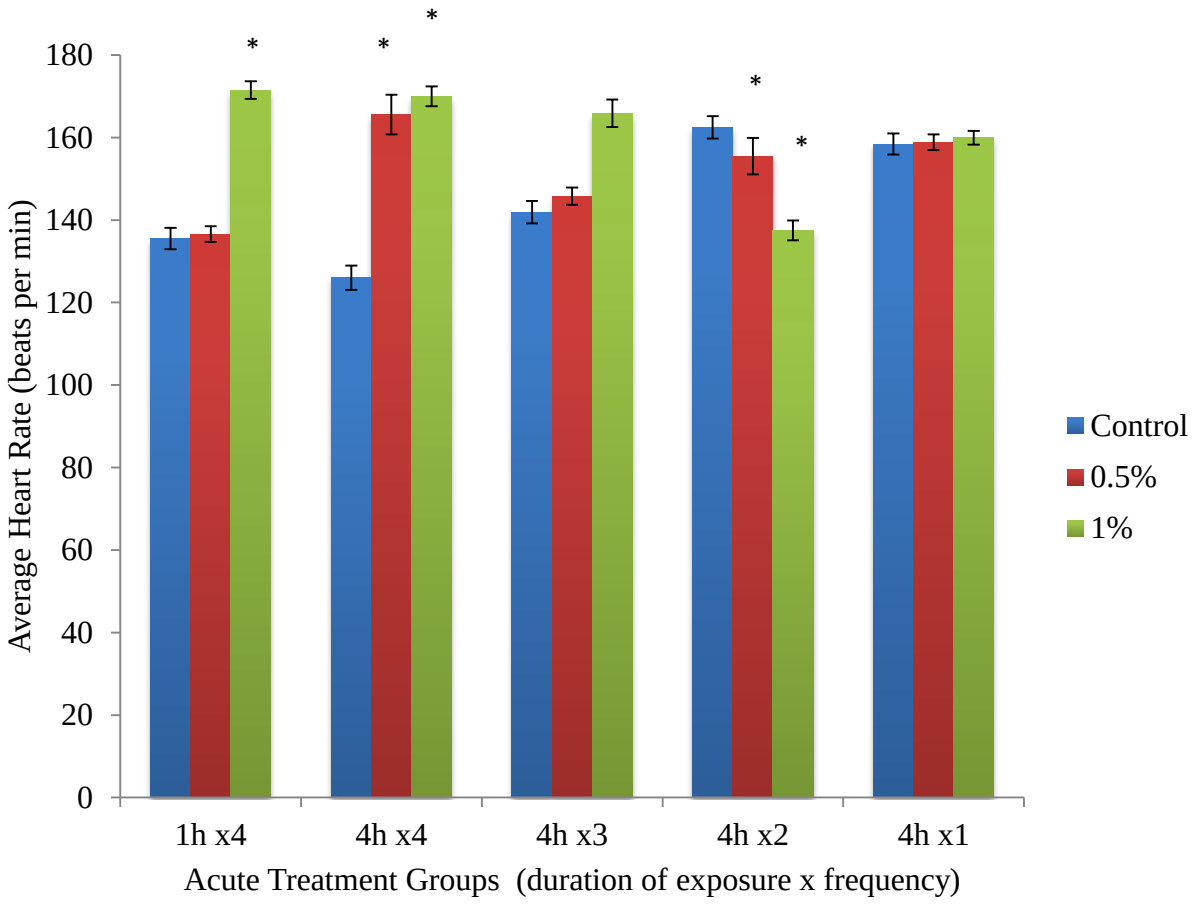

Figure 8. Average heart rates (beats per min) of hatchlings in five acute treatment groups (bars reflect +/- 1SE).

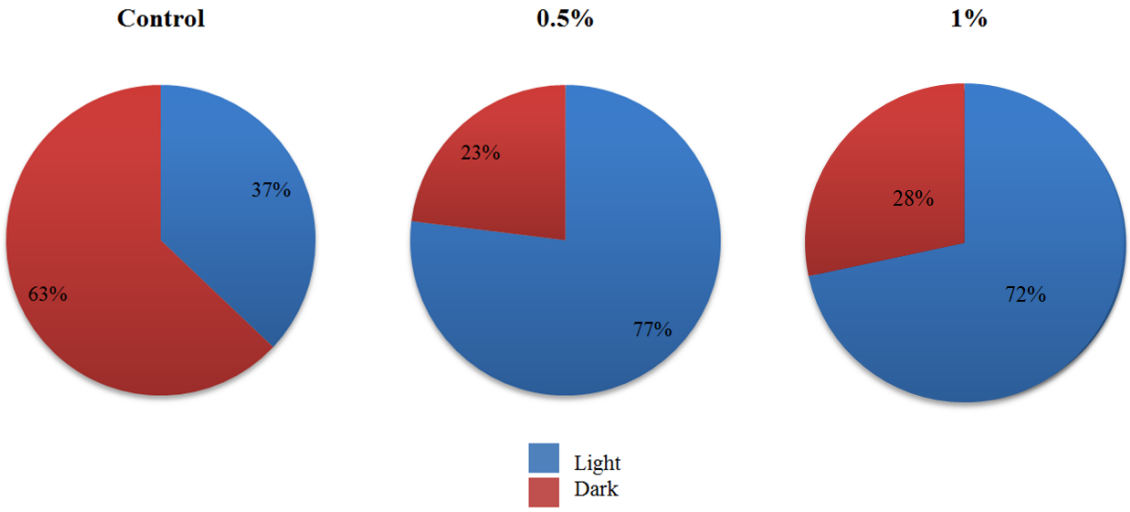

Figure 9. Diagrams of the average percent times the focal zebrafish (n = 3, 3, 2) from respective exposure regiments of the 10-24 hpf treatment groups spent in the lit or darkened portion of the experimental tank.

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