Analysis of the Effects of Alcohol Exposure at Varying Concentrations on *Danio rerio*

Introduction

Zebrafish *(Danio rerio)* are small tropical freshwater fish that have become an increasingly popular model organism in biomedical research. They are about 2-4 cm long when fully mature and fully transparent during developmental periods (Meyers, 2018). This transparency allows researchers to easily observe internal organs and other various tissue structures as they progress through development. One of the more notable advantages zebrafish provide as model organisms is that their genome has been fully sequenced, making them extremely useful for experiments that look to determine genetic expression. With a fully sequenced genome, researchers can use lab sequencing and online database information to help pinpoint exactly which genes may be the cause of observed phenotypes (Adhish & Manjubala, 2023).

In order to properly understand the developmental abnormalities in this study we must first preview the developmental timeline of these organisms. Zebrafish progress from embryo into free swimming larvae in ~72 hours and reach full adulthood in as little as 3 months (Meyers, 2018). The embryonic development is categorized into 5 periods known as; the Zygote Period, where the chorion separates from the yolk, a blastodisc forms and then begins mitotic division. The Cleavage Period, where cleavage and mitotic division continues until 64 blastomeres are produced. The Blastula Period, where the blastodisc forms a spherical structure that sits on the yolk, the yolk syncytial layer is formed, and epiboly is initiated. The Gastrula Period then begins at 50% epiboly where the germ ring appears via involution and the germ layers begin differentiation. The Segmentation Period follows and is defined by the process of somitogenesis where

symmetry begins to take form and the building blocks for bone formation are laid. The Pharyngula Period is characterized by the formation of pigment, fins, and circulatory systems. Finally, the Hatching Period begins and is defined by the development of a more well-developed heart, mouth formation, hair cell differentiation, and final touch eye formation (Meyers, 2018).

Zebrafish reproduce in large numbers with mating sessions resulting in as many as 350 eggs with a typical spawning success of 50% and an average of 100 fertilized eggs (Adhish & Manjubala, 2023). This high end offspring production allows scientists to perform experiments and obtain data on a generational time scale in a matter of months. Zebrafish share many physiological and genetic similarities to humans, including the development of similar organs and tissues such as liver, heart, and musculature (Adhish & Manjubala, 2023). These phenomenal fish are commonly used to study diseases such as cancer, fetal alcohol syndrome, heart disease, and neurodegenerative disorders.

Fetal Alcohol Syndrome Literature Review

Fetal alcohol syndrome (FAS) is a condition that develops when infants are exposed to high levels of teratogens during fetal development. FAS causes physical, cognitive, and behavioral problems that can last a lifetime. The effects of FAS can vary widely, but commonly consist of a few trademark abnormalities. These include; facial abnormalities such as small eyes or decreased intraocular distance, a thin upper lip, and overall small head circumference. Growth deficiencies and inability to reach proper development benchmarks with height and weight. Neurological and cognitive impacts like memory

deficits and difficulty with abstract reasoning and problem-solving. Finally, behavioral symptoms are also commonly seen, such as poor impulse control and social skills (Pinheiro-da-Silva & Luchiari, 2021). The need to better understand FAS and related symptoms is extremely important for biomedical research. If we can grow our foundational knowledge of the mechanisms behind FAS then we can move towards a future with better prevention, better therapeutics, and ultimately better patient outcomes (Pinheiro-da-Silva & Luchiari, 2021).

FAS is caused by the toxic effects of teratogens, and although the overall effects are not completely understood, modern research has been to unlock several mysteries. First and foremost we know that FAS has a direct impact on cell migration and proliferation (Pinheiro-da-Silva & Luchiari, 2021). Since cells migrate to form the germ layers which then lead to tissue differentiation, we know that it's the interference of this process that causes a lot of the abnormal tissue development (Meyers, 2018). Disruption of gene expression is another effect that is well documented (Adhish & Manjubala, 2023). The interference of nutrient and oxygen delivery promotes oxidative stress promoting the production of reactive oxygen species which can damage cells and developing tissues (Fernandes & Lovely, 2021). Research has also been able to determine that FAS causes a neurotoxic environment which leads to inhibition of neurotransmitter signaling (Baggio et al., 2020). Overall, the effects are extremely complex and multifaceted and our understanding of them is still being established.

Several model organisms are being used in the study of FAS. Mice are commonly used because of the direct mammalian relationship they have with humans. However, one major drawback or limitation to mice is that they take up to 3 weeks to

develop from embryo to pup and they are not transparent making it more difficult to observe developmental stages (Bhatia et al., 2019). In contrast, zebrafish reproduce and develop very quickly, sitting only 72 hours from embryo to free swimming larvae. Due to their transparency, researchers can observe various developmental stages and better pinpoint when the effects of FAS begin to take hold in certain tissues. A potential limitation of zebrafish is that the embryos are not developed within a placenta and instead grow within a chorion outside of the mothers body (Meyers, 2018). Nevertheless, they still seem to be the go-to model organism for studying FAS.

Experimental Objective

The objective of this study is to utilize zebrafish embryos as model organisms for the analysis of varying degrees of FAS abnormalities. By observing morphology and recording findings at different developmental stages we compare and investigate variations in abnormalities produced by FAS. This allows for the identification of commonly found symptoms stated in the literature and the subsequent linkage of those symptoms to their respective exposure scenarios/solutions concentrations. Zebrafish embryos are split up into three separate groups, one control and two experimental groups. Experimental groups 1 and 2 are exposed to ethanol solutions for 25 minutes at a time on four separate days. Experimental group 1 is exposed to a .5% solution and experimental group 2 is exposed to a 1.5% solution. We suspect that if the two experimental groups undergo comparative analysis throughout the experimental period, then we will find more prominent FAS derived abnormalities and symptoms in Exp Group 2 than in Exp Group 1.

Materials

All materials below are referenced as provided by Carolina Biological.

Zebra Fish # 145562

Methylene Blue # 875911

Ethanol Item #: 861281

Zebra Fish Breeding Tank Item #: 161937

Sterile Transfer Pipettes Item #: FAM_214551

Petri Dishes, Pack of 12 Item #: 721132

Labels, Notebook

Wolfe® CFL Educational Microscope Item #: 59095

Methodology

Zebrafish Husbandry & Breeding

Water for the zebrafish holding tanks is purified through reverse osmosis systems and then salts and pH buffers are added to ensure optimal water quality. A recirculation system that filters and sterilizes dirty water before returning to the tank is utilized in order to reduce water usage and streamline tank care. Tanks are also covered to reduce evaporation and to keep the fish from jumping out. The zebrafish are fed a combination of dry food and small organisms that mimic their natural diet of zooplankton and insects. Larval and juvenile fish are fed live microorganisms like paramecia, while adult fish were fed commercially available powders and small crustaceans such as brine shrimp. The fish were fed 2-3 times and water recirculation was turned off to prevent the

food from floating away. Tanks were labeled accordingly so that similar genetic backgrounds can be properly accounted for (Jove, 2018).

Embryo Collection

Zebrafish crosses were set up in special tanks designed for mating which have a removable false bottom to catch the embryos. Adult zebrafish were combined in the breeding tanks in the afternoon as this is the most likely time for the fish to breed (Meyers, 2018). A divider was put into place to separate the fish until the following morning providing them ample times to gain comfortability. In the morning, the divider was removed to initiate spawning. After waiting for the males to release sperm into the water for fertilization the adults were removed from the tank and the embryos were collected with a strainer. The embryos were then washed with egg water and transferred to petri dishes, where they were kept in an incubator at 28℃. Once the embryos had entered the 256cell stage, the embryonic experimental procedures began.

Solution & Tool Preparation

The formula C1V1 = C2V2 was used to determine the necessary concentration in ml needed to create the .5% and 1.5% alcohol solutions. A micropipette was then utilized in order to measure the exact amount of pure ethanol needed for the two solutions and a graduated cylinder was used to measure the appropriate amount of egg water. .25 ml of ethanol was transferred into a beaker of 49.75ml of egg water to produce the .5% solution, and .75 ml of ethanol was transferred into a beaker of 49.25ml of egg water to produce the 1.5% solution. Both beakers provided enough solution for the entirety of the

experiment. Egg water was prepared by supervisors prior to the experiment by combining purified water, stock salts, pH buffers, and methylene blue to prevent unwanted fungal or bacterial growth. Egg water was provided in safety squeeze bottles. Next, two sterile transfer pipettes were marked in accordance to their designated use, red for alcohol solution and blue for egg water. Finally, three petri dishes were labeled as the following; Control, .5%, 1.5%.

Experimental Procedure

60 embryos were divided evenly (20 embryos each) amongst 3 separate petri dishes by gently pipetting the embryos into the blue pipette from a 1-2 mm distance in order to avoid damaging them. Before each experimental session the embryos underwent microscopic observation in order to identify developmental changes and morphological abnormalities. Then, the experimental groups were exposed to their respective solutions for 25 minutes in all 5 experimental sessions. Exposure time was calculated by a standard timer application with an alarm. After exposure, the alcohol solution was removed via the red alcohol pipette. The petri dish was then refilled with egg water which was subsequently removed with the red alcohol pipette again. This process was carried out a total of two times after each alcohol exposure for both experimental dishes in order to ensure the embryos and the petri dish were adequately washed of any remaining alcohol solution. Finally, the experimental dishes were refilled with egg water which remained until the following experimental session/day. The used egg water and alcohol solution was discarded into a biohazard container. The control dish was not exposed to any alcohol solution. Data was collected in a lab notebook where daily logs

were kept. Finally as per NIH guidelines, proper euthanasia procedures were carried out accordingly. Zebrafish were exposed to 6.15% sodium hypochlorite solution which was further diluted with 5 parts water to 1.23% overall concentration, for at least five minutes prior to disposal to ensure death.

Data & Observations

Charts/Infographics of Experimental Data

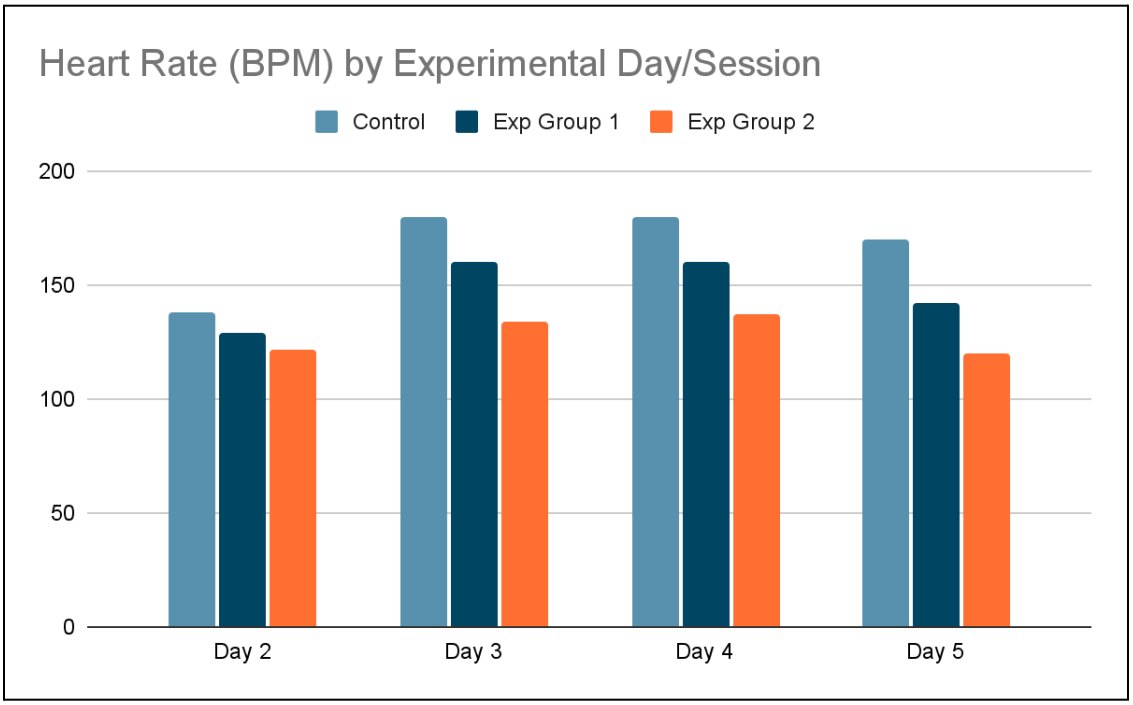


Figure 1. Column Chart represents; Heart Rate in Beats per Minute (Y axis) over Experimental Day/Session (X axis).

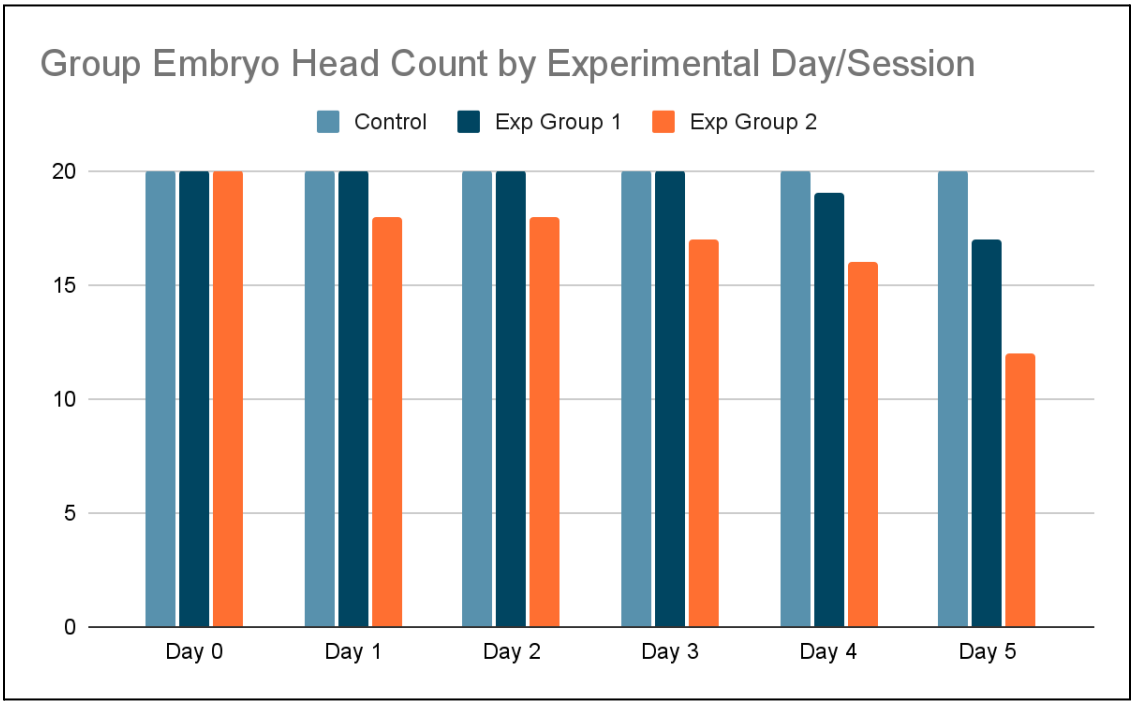


Figure 2. Column Chart represents; Group Embryo Head Count (Y axis) over Experimental Day/Session (X axis).

Gross Data & Observation Notes

Day 0	
Control Group	20 embryos identified as blastula period (2.25-5.25 hpf) and most closely resembling the 256 stage.
Exp Group 1 (.5%)	20 embryos identified as blastula period (2.25-5.25 hpf) and most closely resembling the 256 stage.
Exp Group 2 (1.5%)	20 embryos identified as blastula period (2.25-5.25 hpf) and most closely resembling the 256 stage.
Observation Notes: On day 0 we took our initial observations and found that all embryos were at or near the 256 stage *(See fig 3)*. A major characteristic that prompted this declaration was absence of a visual Yoke Syncytial Layer. In addition, we found oblong morphology or epiboly initiation taking place. This, alongside the fact that the cell count was beyond visual parameters, led us to the overall conclusion.	

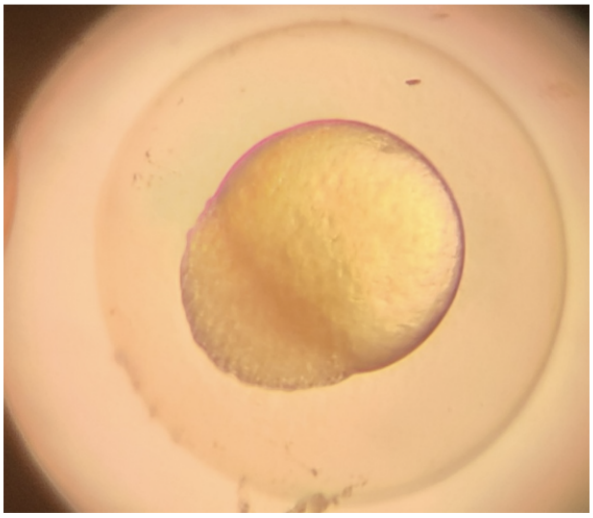


Figure 3. Embryo from the control group on Day 0 reflecting the morphology of the 256 stage.

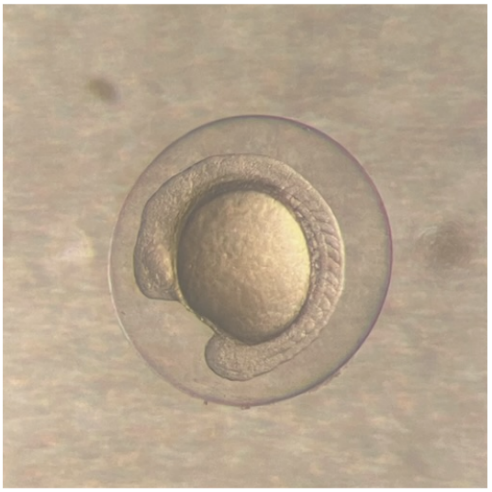


Figure 4. Embryo from the Exp Group 1 on Day 1 reflecting the morphology of the 13 somites stage. Somites appear along the back side of the organism and are within visual parameters for counting

Day 1	
Control Group	20 embryos identified as segmentation period (10-24 hpf) and most closely resembling the 13 somites stage.
Exp Group 1 (.5%)	20 embryos identified as segmentation period (10-24 hpf) and most closely resembling the 13 somites stage.
Exp Group 2 (1.5%)	2 dead embryos were discovered and removed from the dish. 18 embryos identified as segmentation period (10-24 hpf) and most closely resembling the 13 somites stage.
Observational Notes: Two deceased embryos in the Exp Group 2 were observed and identified by the darkened color and lack of discernable shape *(see fig 5)*. Development of all three groups was noted as being slower than expected, it was determined that the most likely cause of this outcome was lower than optimal temperatures in the environment (classroom) at ~20℃. The 13 somites stage was ultimately determined based on time since fertilization and the direct counting of the visible somites *(see fig 4)*. No other morphological or developmental abnormalities were observed.	

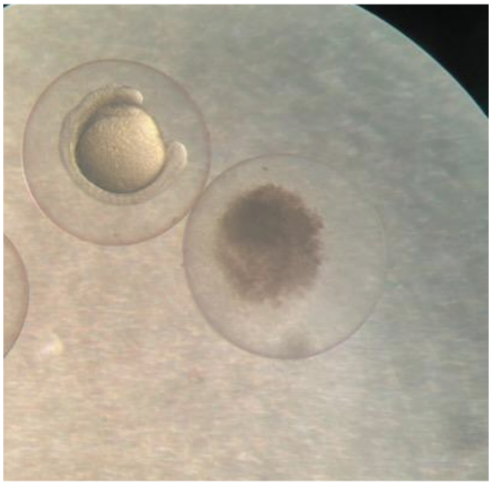
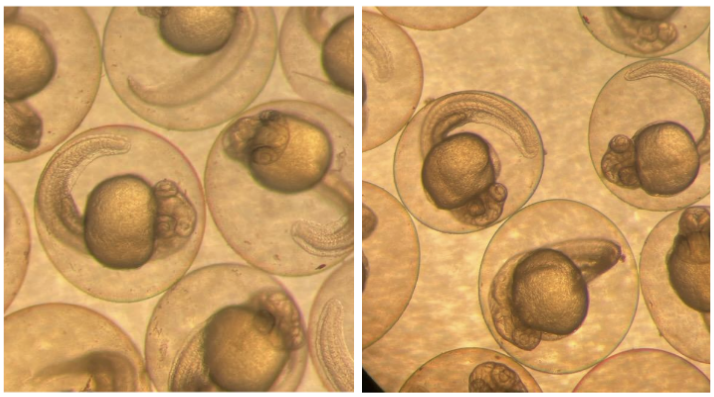


Figure 5. Embryos from the Exp Group 2 on Day 1 with the organism on the right reflecting The common appearance of a dead embryo.



Control Group *Exp Group 2*

Figure 6. Embryos from the Exp Group 2 and Control Group on day 2 showing overall normal development. 25 somite stage is within visual parameters; can be seen and counted clearly.

Day 2	
Control Group	20 embryos identified as segmentation period (10-24 hpf) and most closely resembling the 25 somites stage
Exp Group 1 (.5%)	20 embryos identified as segmentation period (10-24 hpf) and most closely resembling the 25 somites stage
Exp Group 2 (1.5%)	18 embryos identified as segmentation period (10-24 hpf) and most closely resembling the 25 somites stage

Observational Notes:
Embryo head count remained the same as Day 1 with no deceased embryos observed *(see fig 2)*. The control group showed response to gentle tactile stimulation reflecting proper nerve and muscular tissue development was taking place. This response decreased in both reaction time and overall magnitude in the experimental groups, with the Exp Group 1 showing a slightly lethargic response and the Exp Group 2 showing no response at all. No other morphological or developmental abnormalities were observed *(see fig 6)*. Tail movement was observed in both the control group and the Exp Group 1, little to no movement was detected in the Exp Group 2.

Heart rate was calculated via microscopic observation and timer for all three groups *(see fig 1)* and reflected the following;
Control - 138 bpm, Exp 1 - 129 bpm, Exp 2 - 122 bpm.

Day 3	
Control Group	20 embryos identified as pharyngula period (24-48 hpf) and most closely resembling the 36 hour stage.
Exp Group 1 (.5%)	20 embryos identified as pharyngula period (24-48 hpf) and most closely resembling the 36 hour stage.
Exp Group 2 (1.5%)	1 embryo deceased. 17 embryos identified as pharyngula period (24-48 hpf) and most closely resembling the 36 hour stage.

Observational Notes:
Embryo head count dropped by one in the Exp Group 2. Control and Exp Group 1 remained the same as Day 2 with no deceased embryos observed *(see fig 2)*. Pigmentation of the eyes and body were present in all groups as well as bilateral symmetry. The median fin fold was noted as present in the control group only *(see fig 7)*. At this stage, tactile response was recorded in all three groups with the control continuing to show rapid response, Exp Group 1 showing slowed response, and Exp Group 2 showing no response at all.

Heart rate was calculated via microscopic observation and timer for all three groups *(see fig 1)* and reflected the following;
Control - 180 bpm, Exp 1 - 160 bpm, Exp 2 - 134 bpm.

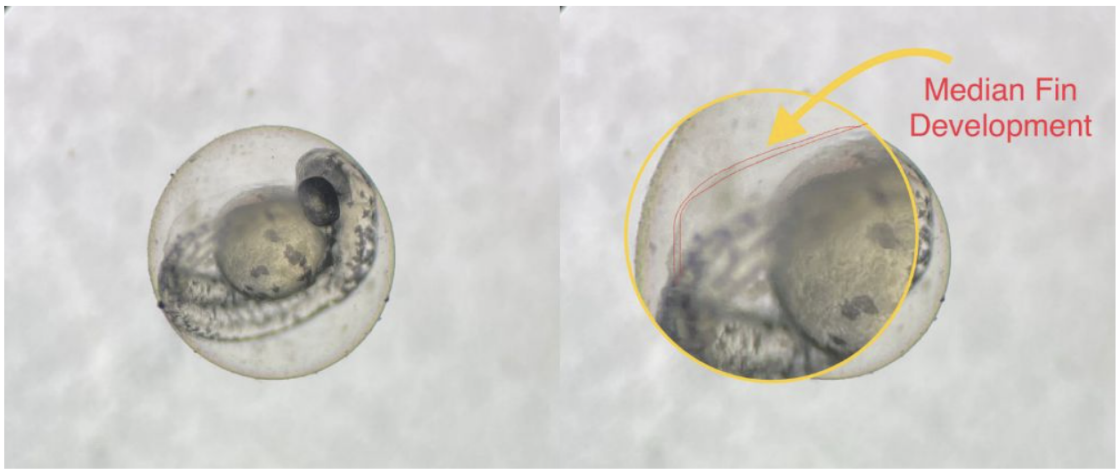


Figure 7. Embryos from the Control Group on day 3 showing median fin development. A hallmark of the 36hr stage.

Day 4	
Control Group	20 embryos identified as hatching period (48-72 hpf).
Exp Group 1 (.5%)	1 embryo deceased. 19 embryos identified as pharyngula period (48-72 hpf).
Exp Group 2 (1.5%)	1 embryo deceased. 16 embryos identified as pharyngula period (48-72 hpf).

Observational Notes:
Embryo head count dropped by one in the Exp Group 1 and 2. Control group remained the same as Day 3 with no deceased embryos observed *(see fig 2)*. Yellow pigmentation of the head was noted in the control group reflecting the development of xanthophores. Control group yolk decreased to half the width of the body. Observed in Exp Group 1 were two hatched larvae and malformed fins. An albino embryo lacking all pigmentation was also noted in Exp Group 1. Two hatched larvae with no fins and chorion covered heads were observed in Exp Group 2 as well as an individual with only one eye and a mid section deformation.

Heart rate was calculated via microscopic observation and timer for all three groups *(see fig 1)* and reflected the following;
Control - 180 bpm, Exp 1 - 160 bpm, Exp 2 - 137 bpm.

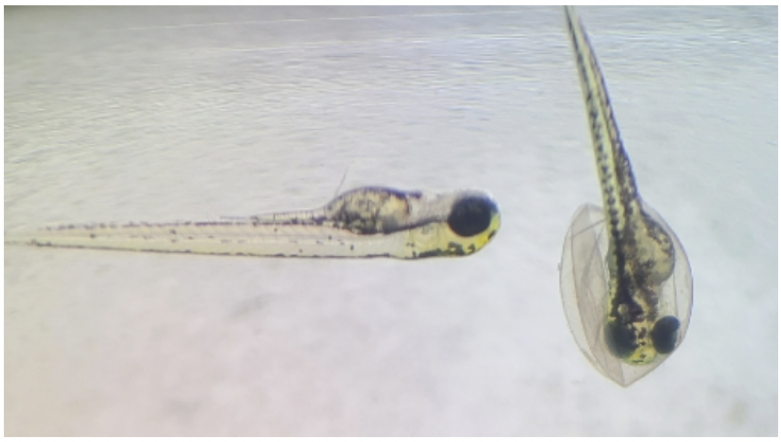


Figure 11. Larvae from Exp Group 2 lacking fin development. Remaining chorion attached to head (R).

Day 5	
Control Group	20 embryos identified as hatching period (48-72 hpf).
Exp Group 1 (.5%)	2 embryos deceased. 17 embryos identified as pharyngula period (48-72 hpf).
Exp Group 2 (1.5%)	4 embryos deceased. 12 embryos identified as pharyngula period (48-72 hpf).

Observational Notes:
Embryo head count dropped by two in the Exp Group 1 and four in Exp Group 2. Control group remained the same as Day 4 with no deceased embryos observed *(see fig 2)*. It was noted that none of the embryos had hatched in the control group. Exp Group 1 larvae were observed having difficulty swimming but no new abnormalities were discovered. In Exp Group 2 several new abnormalities were observed and documented such as a curved (c-shaped) body, misshapen eyes and decreased intraocular distance *(see fig 8)*, lacking fully developed tails, no visual pigmentation, enlarged heart, and complete malformation of body structure.

Heart rate was calculated via microscopic observation and timer for all three groups *(see fig 1)* and reflected the following;
Control - 170 bpm, Exp 1 - 142 bpm, Exp 2 - 120 bpm.

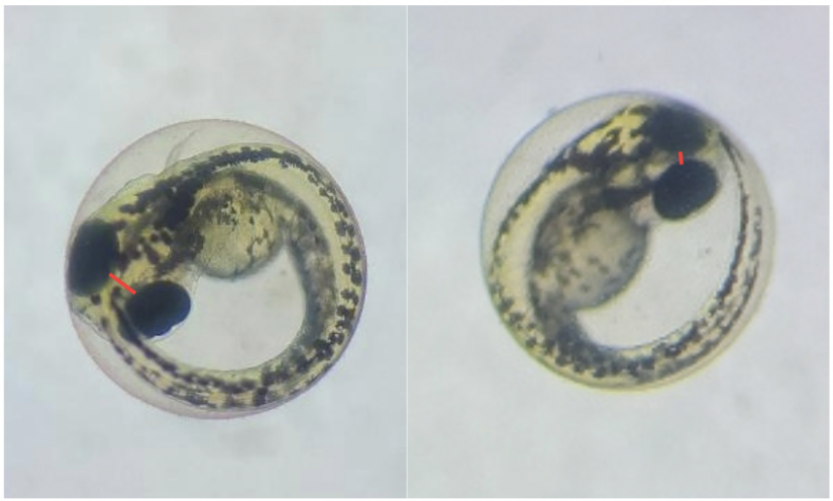


Figure 8. Variation in intraocular distance based on strength of solution concentration. Showing Exp Group 1 (L), and Exp Group 2 (R).

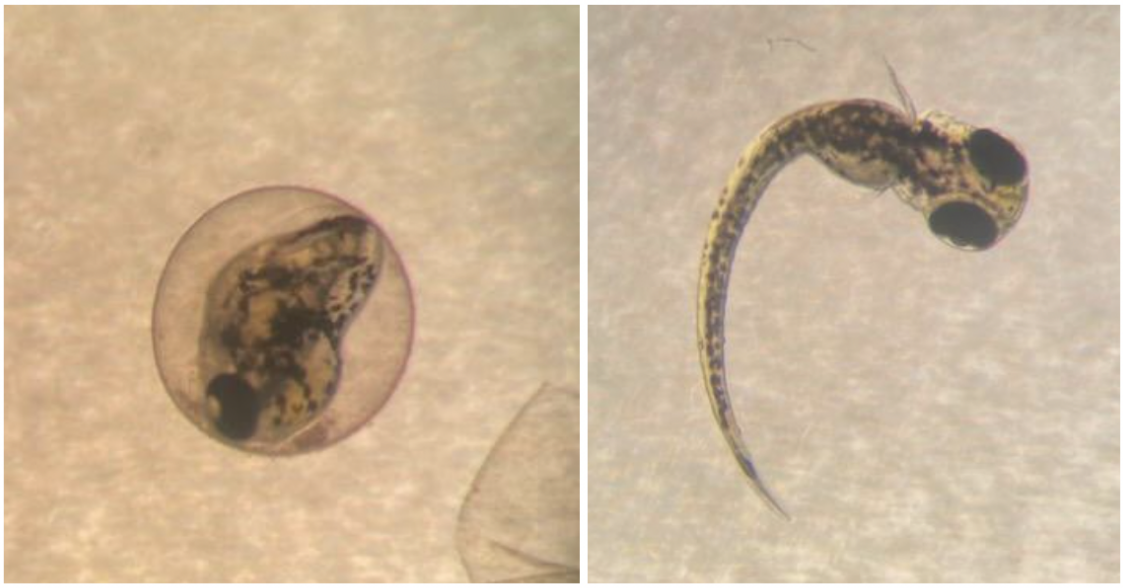


Figure 9. Larvae from Exp Group 2 on day 5. Complete malformation of body structure (L). C-shaped body and missing right fin abnormality.

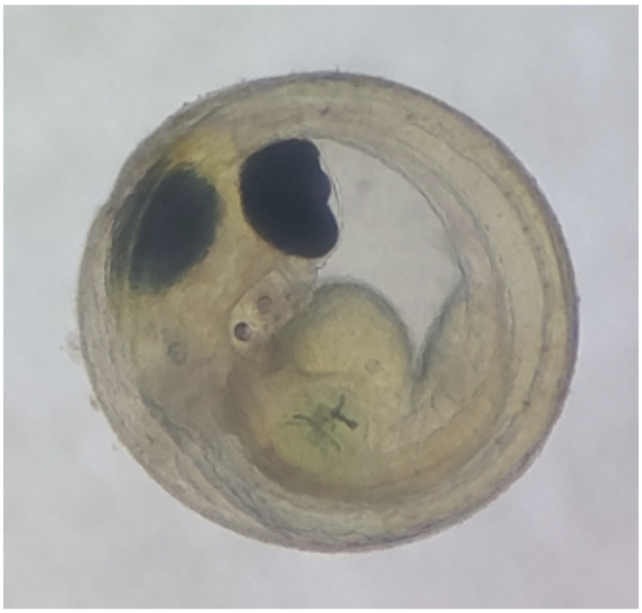


Figure 10. Embryo from Exp Group 1 on day 5. Showing mostly complete development, but lacking all pigmentation aside from the eyes.

Discussion

An overtly noticeable trend that emerged was the embryo death that increased with strength of alcohol solution. As seen in *fig 2*, the Exp Group 2 saw a massive drop off in head count on the final day of experimentation. Considering that the trend began to reflect exponential values, it's likely that if the experiment had continued for a few more days, the majority if not all of the embryos would have died. Another trend that emerged was the variation in heart rate between the groups. Once again we saw that the heart rate decreased with increasing solution strength *(see fig 1)*. The most noticeable phenotype of all was the lack of fin development and poor overall body structure (such as c shaped body and asymmetry) in the Exp Group 2 *(see fig 9),* and to a slightly lesser degree in the Exp Group 1. This could easily be the result of poor germ layer formation or even more specifically, disrupted cell migration during the epiboly stages. If the cell migration was altered due to the alcohol exposure such as the outcomes found in Adhish & Manjubala, 2023, then it would cause a less robust differentiation of the germ layers and ultimately account for the phenotypes observed.

The slight variation in magnitude of developmental malformation between the Exp Group 1 and Exp Group 2, well highlighted in *figure 8,* is in line with the heart rate and premature death trends previously pointed out and thus stands to be well supported by the evidence. A single embryo from the Exp Group 1 deserves a special mention due to its mostly proper development yet complete lack of pigment *(see fig 10)*. Aside from the pigment, the only other noticeable difference was that the embryo had not hatched like many others. Although, as pointed out in Pinheiro-da-Silva & Luchiari, 2021, many embryos will reach late stages of development while remaining in the chorion, making

hatching on its own a poor key indicator of developmental stage. It's possible that this embryo's exposure to alcohol resulted in the death or alteration of a gene which encodes for pigmentation early on in development. A challenge that we faced during the experiment was properly identifying phenotypes in the pre pharyngula periods. In hindsight, it seems probable that we may have been able to identify certain indicators of malformation during the segmentation period such as asymmetries and lack of organ development. In addition, the 48 hour window between day 3 and day 4 is believed to have been the cause of many developmental observations missed at the transitional points from '24-48 hr' and '48-72 hr' stages.

Conclusion

Our hypothesis of increased FAS abnormalities in Exp Group 2 when compared to Exp Group 1 was overwhelmingly supported by almost every metric. We saw this variation in heart rate, embryo death, and morphological malformation. Based on this study, a more thorough analysis of the exact stages affected should be conducted. This study provides a quality framework for reference, but a more in depth comparison of the developmental variations would be useful. For instance, a marking and tracking of specific organ or tissue structures would have allowed for more quantitative analysis of the variations observed. In addition, like so many that have preceded it, this study should serve as a call to action for biologists and young scientists looking to make a difference in the study of developmental diseases such as FAS. This study exemplifies how accessible research in this field can be when utilizing model organisms such as zebrafish.

Future studies should strive to conduct similar experiments but with more experimental groups in smaller increments of solution concentration. This amendment paired with a longer observation period or overall experimental window would provide new insights into the observations recorded here. With a system that includes smaller solution increments, future researchers could better understand how solution variation may effect exactly when specific developmental inhibitions or alterations take place. For instance, if it can be determined that a common abnormality begins to reflect its phenotype at the 25-somite stage for a 1.2% solution, then the potential for that phenotype to appear at a later stage in a 1.0% solution can be tested for.

Citations

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