Sabrina Huang

Brookfield Central High School

Abstract

This paper views the effects of ethanol and caffeine on zebrafish embryos. Zebrafish are used as models because they are genetically similar to humans. Data is used to compare effects of ethanol and caffeine on humans. In this lab, it was observed that when embryos are placed in caffeine and ethanol solutions of all different concentrations, the embryos would die off or be born with problems. All were viewed and compared to a control well with embryo solution. This allows for a comparison between the impacts of the solutions. Two wells were used as control groups. Caffeine solutions, which are found in many daily foods and liquids, concentrated at 0.05 mg/mL, 0.25mg/mL, and 1.0mg/mL were all placed in wells that contained 8 zebrafish embryos. Two wells of 0.25mg/mL and two wells of 1.0mg/mL. Lastly, ethanol solutions, which are commonly found in alcohol or medicine, concentrated at 30mM, 100mM, and 300mM were placed in wells that contained 8 zebrafish embryos. There were two wells of 100mM and two wells of 300mM concentrated solutions. During the seven day experiment, slow development, different spinal cords, and strange movements were present between the two solutions. Each day was recorded and marked. Deaths of embryos were taken along with the number of hatched embryos. These examinations led to the conclusion that caffeine and ethanol damage the progression of zebrafish embryos and can ultimately shorten their lifespan. This conclusion is a way to see the end result of solutions on humans.

Effects of Ethanol and Caffeine on Zebrafish Embryo Models

Zebrafish embryos were used in the experiment to determine the effects of ethanol and caffeine exposure. The purpose of this experiment was to figure out what were the effects of caffeine and ethanol in zebrafish embryos. This data will be used to answer this question and will be compared to possible outcomes of the same chemicals on the embryos to model human health. Over the years, scientists have used numerous types of vertebrates to exemplify humans. Scientists had begun to believe that vertebrates were harder to use than invertebrates because they are less receptive. Fortunately, George Streisinger recognized that even though zebrafish are vertebrates, they have many advantages that invertebrates do not offer including being easy to work with. Zebrafish hold advantages such as high production of eggs, short lives, quick growth, translucent embryos, and easy maintenance (Browder & Iten, 1998). Beside easy access and care, zebrafish are genetically similar to human embryos. Zebrafish are better models than models from the past such as insects and worms that lack a backbone. These advantages are vital factors for researchers to distinguish the issues of ethanol or caffeine used on zebrafish embryos. These factors have made it easier for scientists to use zebrafish as models, thus, it was decided that zebrafish are the best way to showcase data when exposed to toxins and toxicants (Browder, Iten 1998).

Ethanol was one of the substances used in this experiment. Ethanol is a clear liquid and is widely used to disinfect. This liquid, when consumed, is absorbed quickly in the gastrointestinal tract (National Center for Biotechnology Information, n.d.). The GI Tract is the main tube that digests food. When ethanol is absorbed by the GI Tract, it is distributed to all parts of the body, which could cause many problems to a human embryo, if used inappropriately such as overdose (National Center for Biotechnology Information, n.d.). When ethanol is consumed, researchers have established the conclusion that the liquid could lead to a disorder called Fetal Alcohol Exposure to embryos. This will most likely have an effect on the development at any stage during a pregnancy. Effects include brain damage, behavioral problems, and slow development (National Institute on Alcohol Abuse and Alcoholism, 2010). Drinking has also been concluded to have a correlation with premature birth, stillbirth, and death (Bailey, Sokol, n.d.). Drinking alcohol must be watched carefully because the end product can be passed down to the baby if it is not controlled. People with this disorder have trouble with daily socializing. They are bound to

make bad decisions and trust the wrong people (National Institute on Alcohol Abuse and Alcoholism, 2010).

Although zebrafish experience less complex repercussions of Fetal Alcohol Exposure, past experiments have suggested similar slow maturation and defects in zebrafish. Previous studies have been discovered that when zebrafish are exhibited to ethanol at an early stage, it would create worse conditions to the fish. This condition is called cyclopia, which is a disorder that is triggered from failure of embryo growth (Mindel, 2001). The main effect that ethanol can provoke is characterized in the eyes. Either there is a narrow eye or no eyes, at all. The exposure is first caused because of the thickened endoderm, one of the primary germ layers, moves to the wrong location (Mindel, 2001). This supports the hypothesis because it was predicted that when the zebrafish embryo is exposed to ethanol, the zebrafish will advance with a major defect. Cyclopia is a serious defect that has been observed to alter the natural development of zebrafish (Mindel, 2001).

The other substance that was used in this experiment is caffeine. Caffeine is found in everyday drinks and foods like coffee, tea, soda, chocolate, and many more. The reason why this drug is found in so many daily items is because of its main use, alertness (March of Dimes, 2015). Sometimes it is combined with painkillers to treat headaches. Caffeine alerts the nervous system which includes the heart, muscles, and organs that control blood pressure (Web MD, n.d.). Since caffeine is such a widely used toxin, it has been rejected as a main concern to pregnancy. In actuality, caffeine has been found to affect a baby during pregnancy. Besides increasing blood pressure, heart rate, and the amount of urine, it may cause a person’s sleep to be problematic. During pregnancy, caffeine can pass through the placenta and cause the baby to be supplied with caffeine (March of Dimes, 2015). Having too much caffeine can lead to death, but this topic is still highly debated and supported by different studies (March of Dimes, 2015). Additionally, higher amounts of consumption pass through to breast milk and cause more problems (Web MD, n.d.).

Similarly to humans, zebrafish consumption of caffeine perturbs the heart rate and can result in death. According to experimentations, it was studied that the response of caffeine on zebrafish embryos changed the heart rate (Institute of Life Sciences, 2010). The subjection to caffeine compelled a reduction in the heart rate of embryos which resulted in the heart rate to stop. Because of this, zebrafish growth would be affected and cause the fish to be born with a

distortion. This experiment relates to our hypothesis by supporting it. The decrease in heart rate, slows down growth. The toxin, caffeine does influence zebrafish development when exposed and will stunt any growth (Institute of Life Sciences, 2010).

Observers hypothesized that if caffeine or ethanol are exposed to zebrafish embryos, then the zebrafish will progress with critical flaws or die because the toxins/toxicants will adjust the natural development of the embryo.

Materials

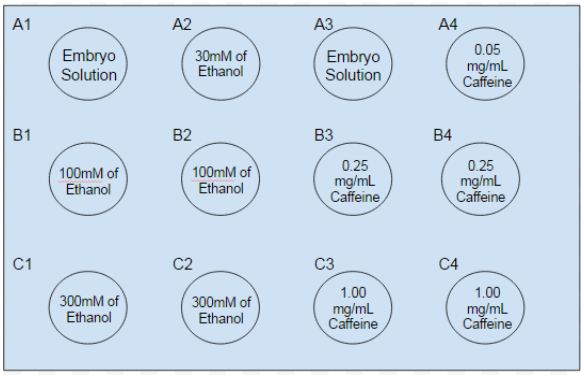
- Dissecting Microscopes
- Zebrafish Embryos
- Embryo Media
- 3x4 Multi-Well Plate
- Disposable Wide Bore Pipette
- Disposable Thin Bore Pipette
- Distilled Water
- Beaker
- 30, 100, 300 mm of Ethanol
- 0.05, 0.25, 1.0 mg/mL concentration of Caffeine
- LGG3 Phone Camera
- IPhone 6 Camera

Procedure

Day 1 -

- We labeled plates and filled each well with 1mL of the appropriate solution, according to *Figure 1*.
- Our initial observations were written down.
- We set the embryos aside on the table and they were NOT put into the incubator.

Figure 1: The order of solutions of each toxin/toxicant in a multi well plate.



Day 2 (24 hours afterwards) -

- We observed embryos and wrote down observations. This includes the number of hatched, dead and any new qualitative development. Afterwards, we took pictures of each well.
- Dead embryos were taken out using a wide bore pipette and dumped into a waste container.
- We changed the old solution with 1 mL of new solution using a thin bore pipette.
- We placed the multi well plate aside on the table.

Day 3 through 7 -

- Steps from Day 2 were repeated.
- Day 5, we took the old solution out and 2mL of new solution was added using a thin bore pipette. Wells were kept over the weekend untouched.

Afterwards -

- We discarded any embryos after last observations.

Results

Overall trends arose throughout both caffeine and ethanol solutions. Weaker concentrations did not have a huge impact on survivals (*Table 1 and 3).* In both ethanol and caffeine, there was no deaths in the weakest concentrations. Hatched embryos increased in both caffeine and ethanol in days 5 through 7 (*Table 2 and 4).* The effectiveness of the two solutions started developing in the same way (*Figure 3 and 6),* but by day 3 or 4, the embryos began to shift in different ways based on the solution. By 24 hours, observations were all similar and most were developing clear fins. By 48 hours, black eyes and a tail had developed but none had hatched yet. By 72 hours, some had developed an orange heart and became more visible to the naked eye. This is about the time where the embryos started to diverge differently from each other.

Embryos in ethanol solutions had a smaller impression displayed than those in caffeine. In the first 24 hours, there were a few fish embryos that had died off, but there was not any consistent pattern (*Table 1).* There was a lack of trends in surviving embryos based on concentration. There was no change in the next six days, as the survival of the embryos stayed constant. The weakest concentration had a slower response on the hatched rate. Contrasting with the slow response, the strongest concentration hatch rate was more than the control's hatched rate (*Graph 2).* In the 100mM wells, the average was 6 hatched embryos and the average of the 300mM was 7. During the stages of hatching, some embryos found in the stronger concentrations behaved differently than the control. Embryos that were different had behaviors that included twitching unusually and a curved spine.

Caffeine solutions showed a stronger and more rapid effect on the embryos. The first 24 hours did not indicate much change, but by the next day the strongest concentration of caffeine had already killed two embryos in both 1.00mg/mL wells. In the next couple of days, both wells with the strongest concentrations had no more living embryos. This trend occurs from day 4-7. Other concentrations stayed constant and did not have much aftermath. Very few embryos died in wells A4, B3, and B4 (*Table 3).* The rate of hatching embryos was lower in all concentrations than the control. The weaker the concentration, the weaker the effect. Any concentrations will lower the hatching rate (*Table 4).*

Table 1: The number of surviving zebrafish embryos of ethanol solutions in a span of seven days.

Treatment	Day 1 (control)	Day 2 (24hrs)	Day 3 (48hrs)	Day 4 (72hrs)	Day 5 (96hrs)	Day 6 (144hrs)	Day 7 (168hrs)
Control (A1)	8	8	8	8	8	8	8
30mM Ethanol (A2)	8	8	8	8	8	8	8
100mM Ethanol (B1)	8	6	6	6	6	6	6
100mM Ethanol (B2)	8	8	8	8	8	8	8
300mM Ethanol (C1)	8	8	8	8	8	8	8
300mM Ethanol (C2)	8	7	7	7	7	7	7

Graph 1: The number of surviving embryos of different ethanol solutions in seven days. It shows a constant rate throughout.

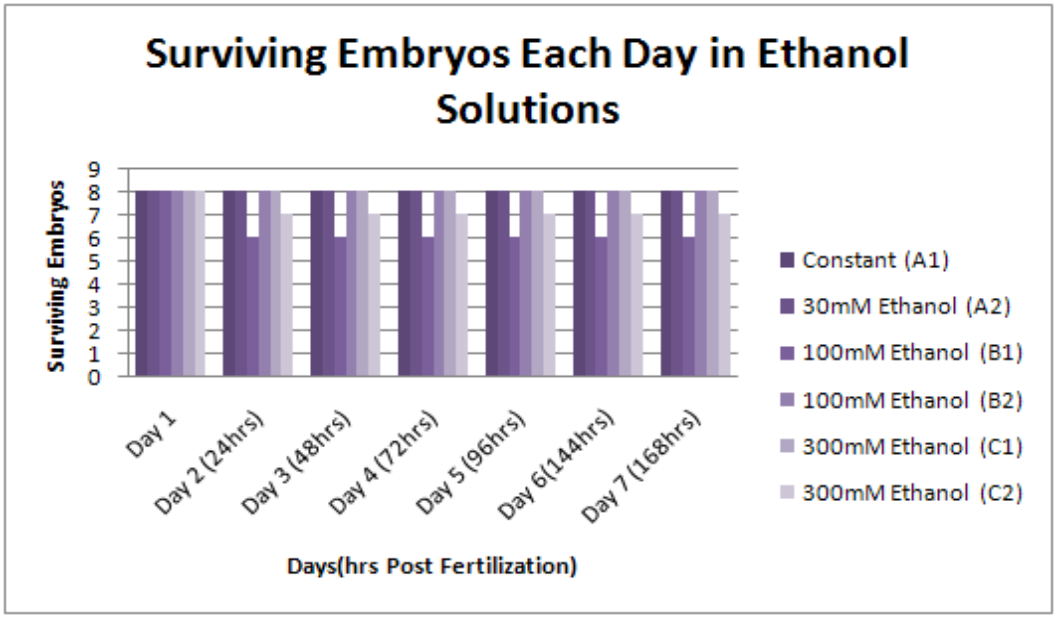


Table 2: The number of hatched embryos for each ethanol solutions in days 5 through 7. Results from previous days are not included because no embryos hatched.

Treatment	Day 5 (96hrs)	Day 6 (144hrs)	Day 7 (168hrs)
Control (A1)	0	2	5
30mM Ethanol (A2)	0	0	2
100mM Ethanol (B1)	1	2	8
100mM Ethanol (B2)	0	1	4
300mM Ethanol (C1)	0	5	8
300mM Ethanol (C2)	0	3	6

Graph 2: The number of hatched embryos increased in the last 3 days of different ethanol solutions. Results from previous days are not included because no embryos hatched.

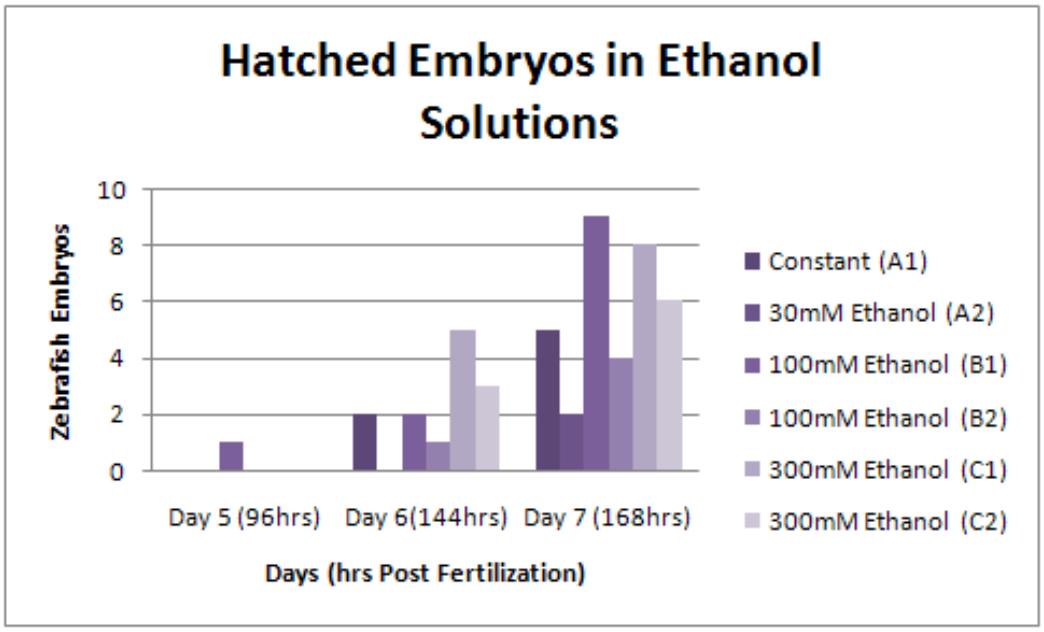


Table 3: The number of surviving zebrafish embryos of caffeine solutions in a span of seven days.

Treatment	Day 1	Day 2 (24hrs)	Day 3 (48hrs)	Day 4 (72hrs)	Day 5 (96hrs)	Day 6 (144hrs)	Day 7 (168hrs)
Control (A3)	8	8	8	8	8	8	8
0.05mg/mL Caffeine (A4)	8	8	8	8	8	8	8
0.25mg/mL Caffeine (B3)	8	8	8	8	8	8	6
0.25mg/mL Caffeine (B4)	8	7	7	7	7	7	7
1.00mg/mL Caffeine (C3)	8	7	5	0	0	0	0
1.00mg/mL Caffeine (C4)	8	8	6	4	0	0	0

Graph 3: The number of surviving embryos drops more drastically in the stronger concentrations of different caffeine solutions in seven days.

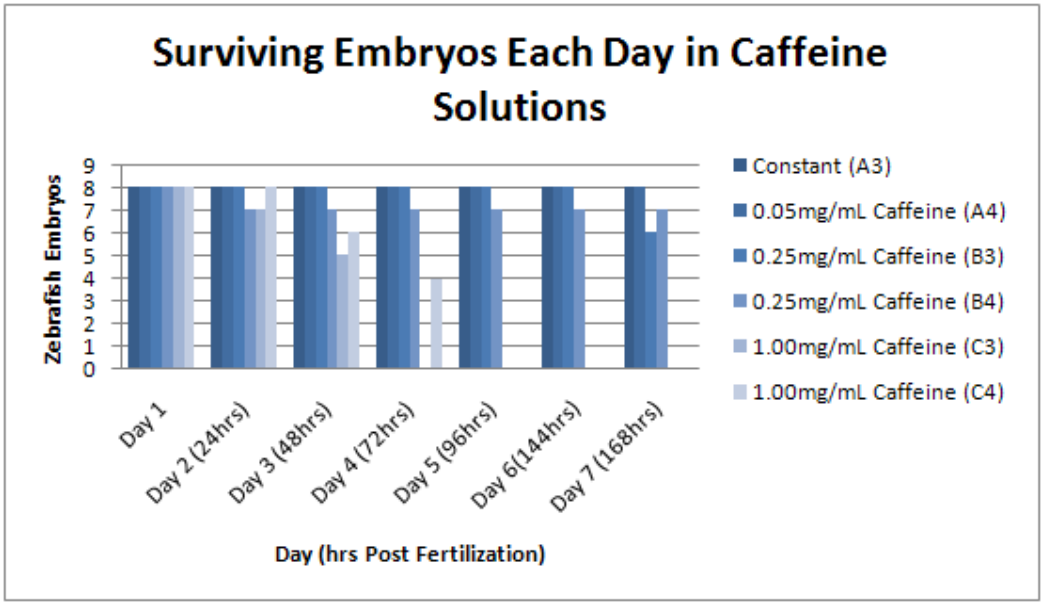


Table 4: The number of hatched embryos for each caffeine solutions in days 6 and 7. Results from previous days are not included because no embryos hatched.

Treatment	Day 6(144hrs)	Day 7 (168hrs)
Control (A3)	4	5
0.05mg/mL Caffeine (A4)	1	4
0.25mg/mL Caffeine (B3)	0	0
0.25mg/mL Caffeine (B4)	1	1
1.00mg/mL Caffeine (C3)	0	0
1.00mg/mL Caffeine (C4)	0	0

Graph 4: The number of hatched embryos slowly increased in the last 2 days of different caffeine solutions. Results from previous days are not included because no embryos hatched.

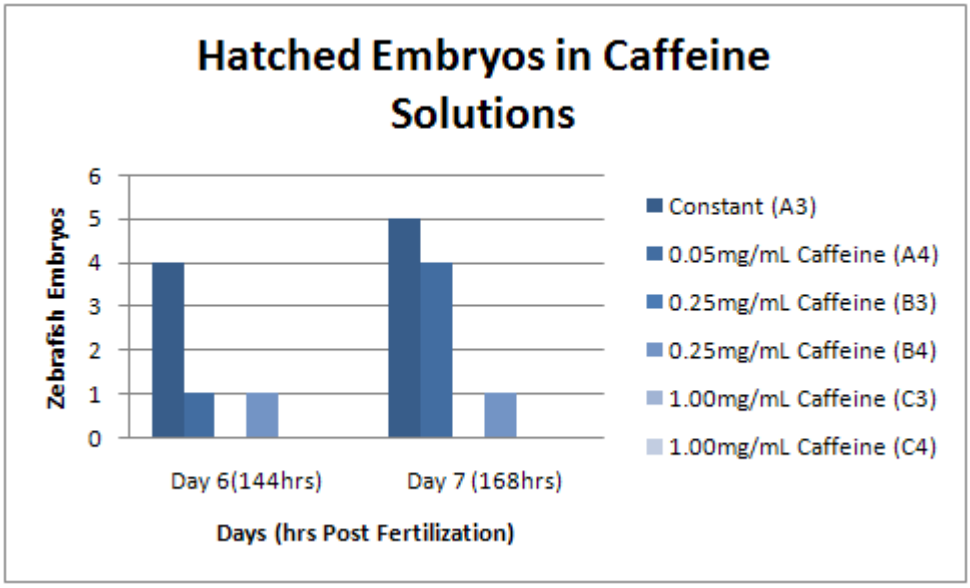


Table 5: Full table of the number of surviving embryos for all the solutions tested in a span of 7 days.

Treatment	Day 1	Day 2 (24hrs)	Day 3 (48hrs)	Day 4 (72hrs)	Day 5 (96hrs)	Day 6 (144hrs)	Day 7 (168hrs)
Control (A1)	8	8	8	8	8	8	8
30mM Ethanol (A2)	8	8	8	8	8	8	8
100mM Ethanol (B1)	8	6	6	6	6	6	6
100mM Ethanol (B2)	8	8	8	8	8	8	8
300mM Ethanol (C1)	8	8	8	8	8	8	8
300mM Ethanol (C2)	8	7	7	7	7	7	7
Control (A3)	8	8	8	8	8	8	8
0.05mg/mL Caffeine (A4)	8	8	8	8	8	8	8
0.25mg/mL Caffeine (B3)	8	8	8	8	8	8	6
0.25mg/mL Caffeine (B4)	8	7	7	7	7	7	7
1.00mg/mL Caffeine (C3)	8	7	5	0	0	0	0
1.00mg/mL Caffeine (C4)	8	8	6	4	0	0	0

Table 6: Full table of the number of hatched embryos for all the solutions tested in a span of 3 days. Results from previous days are not included because no embryos hatched.

Treatment	Day 5 (96hrs)	Day 6(144hrs)	Day 7 (168hrs)
Control (A1)	0	2	5
30mM Ethanol (A2)	0	0	2
100mM Ethanol (B1)	1	2	8
100mM Ethanol (B2)	0	1	4
300mM Ethanol (C1)	0	5	8
300mM Ethanol (C2)	0	3	6
Control (A3)	0	4	5
0.05mg/mL Caffeine (A4)	0	1	4
0.25mg/mL Caffeine (B3)	0	0	0
0.25mg/mL Caffeine (B4)	0	1	1
1.00mg/mL Caffeine (C3)	0	0	0
1.00mg/mL Caffeine (C4)	0	0	0

Figure 2: Under developed murky embryos found in C4, 1.00mg/mL of ethanol at 72 hours.

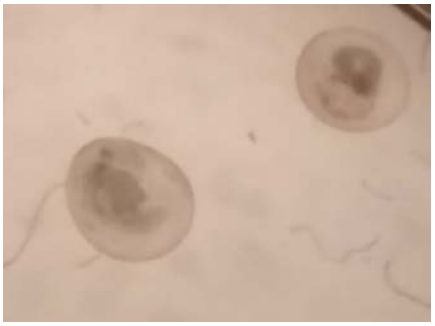


Figure 3: An embryo developing a spotted tail with dark bulging eyes found in B3, 0.25 mg/mL concentration of caffeine at 96hrs.

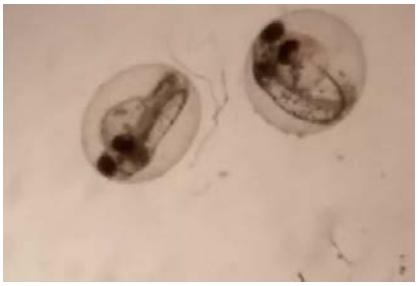


Figure 4: A murky embryo that is half developed found in B1, 100mM of ethanol at 96 hrs.

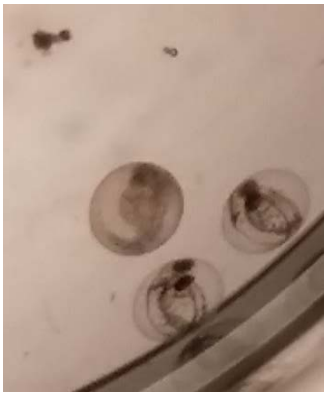


Figure 5: Hatched zebrafish found in B1, 100mM concentration

Figure 6: Developing embryo found in A3, controlled. solution.

Conclusion

Before the experiment, it was hypothesized that if there is exposure to caffeine or ethanol to the embryos, then the zebrafish would develop with malformations or die because the solutions would change the maturation. Our experiment supports our hypothesis based on the observations and data collected.

During this experiment, there were many conclusions that can be drawn out. It was found that there was a higher average of hatched embryos in 100mM of ethanol than the control (*Graph 2).* This data backs up the conclusion that hatched rates in stronger concentrations can speed up growth which may end in a premature death. This conclusion can be used as a prediction for humans using these solutions. Our experiment clearly correlates with the statement that humans who drink ethanol commonly, can give birth prematurely which can lead to problems. Unfortunately, data collected on the survival of embryos in ethanol solutions did not reveal any correlation. Mutations and deformations included a curved spine and unusual twitching which coincides with data found in humans of behavioral problems. Caffeine solutions presented a much clearer correlation. In wells C3 and C4, 1.00 mg/mL caffeine concentrations generated deaths of embryos much quicker than any other treatments (*Table 5).* After just 72 hours, undeveloped embryos were found dead in the well (*Figure 2).* This concludes the stronger the caffeine concentration, the faster for embryos to die before birth. It can also be concluded that caffeine lowers the chances of hatching. The stronger the concentration of caffeine, the lower chances of hatching (*Table 6).* Shockingly, caffeine engendered bigger problems than ethanol.

Humans who drink caffeine too frequently can result in deaths before birth, just like the zebrafish, lowering the chances of survival. It can be concluded that both solutions do have an effect that alter the natural development in embryos. These effects cause death or premature births. In *Table 6,* the control wells had five embryos each, while caffeine solutions had less and ethanol solutions averaged out to have more.

One problem that occurred during this experiment is the use of the wide bore pipette. If not careful, zebrafish embryos could have been sucked up into pipette. This can prompt in broken back which can be mistaken as a deformity. This can also hurt the growth of embryos which hides which variable is disturbing the outcome. Another error that could have occurred is the possibility of incorrect data collection. This can alter the data which leads to a bigger percent error. Some ways of improving this experiment is being more cautious of changing solutions. Having multiple trials is another solution that could eliminate disrupted data. By being more careful and having more trials, this can increase the accuracy. This would also decrease the chances of having lurking variables that could lead to different results.

Additional questions brought up included, would a stronger correlation show if a stronger concentration was used and how does the chance of survival change in different temperatures? If so, why would temperature affect it.

Works Cited

Bailey, A., Beth, Sokol, J., Robert. (n.d.). *Prenatal Alcohol Exposure and Miscarriage, Stillbirth, Preterm Delivery and Sudden Infant Death Syndrome.* http://pubs.niaaa.nih.gov/publications/arh341/86-91.htm

Browder, L., Iten L., (1998, March 4). *Zebrafish as a Developmental System.* (n.d.). Retrieved from January 29, 2016 http://people.ucalgary.ca/~browder/virtualembryo/why_fish.html

Institute of Life Sciences. (2010, March 7). *Caffeine Induced Effects on Heart Rate in Zebrafish Embryos.* Retrieved January 29, 2016 from http://www.ncbi.nlm.nih.gov/pubmed/20415645

March of Dimes. *Caffeine in Pregnancy.* (October 2015). Retrieved February 21, 2016 from http://www.marchofdimes.org/pregnancy/caffeine-in-pregnancy.aspx

Mindel, J. (2001, Aug. 1). *Effect of Ethanol on Zebrafish Development.* Retrieved January 29, 2016 from http://www.swarthmore.edu/NatSci/sgilber1/DB_lab/Fish/Ethanol_1.html

National Center for Biotechnology Information. (n.d). *Ethanol.* Retrieved February 19, 2016 from http://pubchem.ncbi.nlm.nih.gov/compound/702#section=Top

National Institute on Alcohol Abuse and Alcoholism. *Fetal Alcohol Exposure.* (October, 2010) Retrieved February 19, 2016 from http://www.niaaa.nih.gov/alcohol-health/fetal-alcohol-exposure

Web MD. *Caffeine.* (n.d.). Retrieved February 21, 2016 from http://www.webmd.com/vitamins-supplements/ingredientmono-979-caffeine.aspx?activeingredientid=979