Effect of Differing Concentrations of Caffeine on Zebrafish Embryo Development

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Abstract

This lab investigates the effect of caffeine on embryonic development, using zebrafish, a fish with organ function and development extremely similar to that of humans. The findings concluded that caffeine does have a large impact on zebrafish development, as many of the caffeine-exposed fish experienced birth defects, abnormal function, or death, and these results were more noticeable as the concentration increased. The results featured curved/abnormal spine growth in zebrafish embryos which were exposed to lower concentrations of caffeine, and death in embryos with higher concentrations of exposure. These results are likely related with the expected results of human embryos when exposed to caffeine, and serves to show the dangers of high levels of caffeine on embryonic development, raising awareness for pregnant females with regular high-concentrations of caffeine.

Introduction

Caffeine is ubiquitous in all parts of society in today's world. It can be found almost everywhere from espressos purchased at businesses to average in-home coffee beans. Most people in the United States have come into contact with caffeine in one form or another. "Up to 400 milligrams (mg) of caffeine a day appears to be safe for most healthy adults" (mayoclinic.org, n.d.). Though it is classed as a psychoactive drug with brain altering properties, the benefits are often valued more than the negatives, a study paper concluded that the "benefits" of caffeine include: "more clear thinking and less fatigue" as well as improved attention (Bolton, Null, 1981). On the other hand, there has been significant research that suggested that prolonged caffeine exposure leads to withdrawal-like symptoms (Juliano, Huntley, Harrel, et al, 2004). A study that oversaw these symptoms noted that when longtime users of caffeine halted their consumption of the substance, they experienced mood disturbances and flu-like symptoms (Juliano, Huntley, Harrel, et al, 2004). Both studies serve to show the effects of caffeine on the whole body during and after consumption.

Perhaps one of the greatest effects caffeine can have on the body is its impact on neural function, which directly influences other body organs and systems. Adenosine is a neurotransmitter in the central nervous system in charge of slowing neural activity and causing effects such as sleep facilitation and dilation of the blood vessels (Dubuc, n.d.). Caffeine acts as an adenosine-receptor antagonist (or competitor) and inhibits the effects of adenosine, therefore causing stimulation/excitement of the nervous system (Dubuc, n.d.), where many of the leading symptoms of caffeine come from. Ingesting caffeine also enacts the release of dopamine, another important neurotransmitter. The release of dopamine increases the likelihood of a person repeating an action. Thus, the release of dopamine correlates with how people become accustomed with caffeine (Sinicki, 2014). Further studies have tried to show how fewer D2 receptors (a dopamine receptor found on the thalamus and other parts of the body) resulted in increased creativity (Sinicki, 2014). Similarly, cortisol, the stress hormone is also released in response to caffeine, which creates symptoms of increased anxiousness (Sinicki, 2014). Along with the release of cortisol, serotonin is also released to counteract the effects of cortisol (Sinicki, 2014) Serotonin works to regulate mood and gives the "good feel" mood to balance the "stress" mood effects of cortisol (Sinicki, 2014).

In addition to the effects of caffeine on the nervous system, caffeine targets key body parts and systems. A study on rats and bone metabolism determined that caffeine plays a role in hurting bone development and led to other mostly negative effects on bone metabolism, "including bone mineral loss, lower BMD, and lower calcium content," leading to an overall impairment of bone formation (Huang, Yang, Hsieh, et al, 2002). The effects of caffeine on calcium are also proven by other sources, claiming that it can, "cause the body to lose calcium, and that can lead to bone loss over time," resulting in a greater chance of developing osteoporosis (Gavin, 2014). Caffeine also proves to be a mild diuretic, causing more frequent urination (Gavin, 2014). Another effect of the popular drug is a moderate increase in blood pressure, especially within the first 15 minutes in intake (Saville, n.d.). However, both of these effects on the kidney prove temporary, and the effects of the caffeine on kidneys and urination are seldom researched. Other interesting studies determining the effects of caffeine on the body show how caffeine can impact skeletal muscle. A study concerning the effects of caffeine on skeletal muscle concluded that exposure to caffeine reduces the time of fatigue greatly among rats, allowing the caffeine-exposed rats to run for up to 60% longer than the control (Stock, Mehl, Buggy, et al, 2002). Another suggestion on the effects of caffeine on muscles entails the details of how caffeine increases the availability of free fatty acids, leading to a higher fat metabolism (helps you burn fat faster), and ultimately sparring muscle glycogen (Stock, Mehl, Buggy, et al, 2002). These results, however, are mixed, and the effects of caffeine on fat metabolism are not yet confirmed. Furthermore, caffeine shows a distinct correlation with miscarriages and infertility (Harvard Medical School, 2015). Studies have shown that pregnant women who have drank at least two cups of coffee (both of which have 200mg of caffeine) could double the risks of miscarriage (Davis, 2014). Higher concentrations of caffeine prove to be consistent with miscarriages, while lower amounts of caffeine (less than 200mg) seem to pose no threat of miscarriage, as no credible data seems to prove lower concentrations harmful (Davis, 2014). Overall, caffeine effects various body organs and can entail a wide array of effects, which could prove more dangerous to certain individuals (in this case, pregnant females).

This lab will focus on some of the major aspects of organ and body development effects caffeine has through the use of zebrafish. This is an important topic to research in order to correlate what the effects of caffeine are with embryonic development in humans, and the exact differences and systems in which caffeine can impact. The use of zebrafish is essential to this experiment because zebrafish development and organ function are "strikingly similar to those of humans" (Santoriello, 2002). By observing the caffeine effects on zebrafish embryos at different concentrations, it will determine what the "safe" concentration of caffeine intake is, and what concentrations cause the most harm to embryonic growth, providing new and necessary information for women whose caffeine intake is high in order to protect their upcoming child from birth defects or death. Embryonic development serves to be an important stage in human growth, by determining the effects of caffeine on parts of zebrafish development, this lab hopes to serve the public in informing what constitutes "safe" caffeine intake for an impregnated female.

Methods and Materials

This lab will be completed by using different concentration exposure (0.05, 0.025, and 1.0 mg/mL Caffeine) to zebrafish among 3 different wells, each containing 10 fish. Observation and pictures will be included to represent the data for the lab. Based on the research accumulated, the lab will likely yield

results that will show a greater number of genetic defects and abnormal function in fish exposed to higher level concentrations of caffeine.

Materials

-1 bottle each per group Stock solutions of Caffeine (0.05, 0.25, 1.0 mg/mL Caffeine)
-1 per group Beaker for dead embryos and liquid disposal
-1 Sharpie
-1 bottle per group Instant Ocean/Embryo Media Solution
-1 per group plus extras Disposable pipette, minimum bore, 1.5 mm for transferring eggs to observation container and manipulating them in the container)
-1 per group Disposable pipette, 1mL
-1 per group Plate with wells
-1 28.5oC Incubator
-1 per group Depression slide with cover slip
-1 per group Dissecting and compound microscope
-1 per group Notebook or other paper to write down notable observations
-1 per group Camera or other lens-device to take pictures

Procedure

Day 1

Spawning tank was set up, and feeding brine shrimp in preparation for spawning was completed.

Day 2

1. Obtain rinsed embryos from your teacher. Source: Donald Harr, Kaitlyn Brahm (Mukwonago High School, Mukwonago, WI)

2. Label your plate with your name and class hour. Label the caffeine concentration of each well using the Sharpie provided.

3. Fill the one well of the plate with 1 mL of Instant Ocean/Embryo Media solution using the disposable pipette. Fill the remaining wells with the appropriate caffeine stock solutions. Divide the embryos so there are approximately 10 embryos in each well. Label the plate on the student data sheet.

4 Record exact numbers of live embryos on student data sheet.

NOTE: Dead embryos should be discarded.

5. Observe your embryos under the dissecting microscope. Record observations on student data sheet.

6. Place each plate in the 28.5OC incubator overnight.

Day 3

1. Remove plate from incubator.

2. Remove dead embryos from the plate using the transfer pipette. Squirt dead embryos into waste beaker. Be careful to only remove the dead embryos.

3. Count remaining embryos, hatched fish, and record in data table.

4. Remove caffeine stock solutions from each well of the plate.

NOTE: Tilt the plate so the embryos settle and remove the liquid from the top.

5. Add the appropriate fresh caffeine stock solution using a clean pipette each time.

6. Place the plate under dissecting microscope and record observations on student data sheet. Note/describe any developmental markers and abnormalities. Repeat for all caffeine concentrations.

7. Remove 1-2 embryos and place on the depression slide with cover slip. Observe the embryos using the compound microscope. Record observations on student data sheet. Repeat for all caffeine concentrations.

8. Return the embryos to their well in the plate.

9. Return the plate to the appropriate 28.5OC incubator.

Day 4

1. Repeat Day 3 work and observations. Record all data.

Safety

In order to ensure that the well-being of lab participants is in though during the lab, and to few rules for safety regulation should be followed. These rules will help guide the ethical conduct of the lab and prevent possible errors and confounding variables to interfere with results.

-Wear closed-toed shoes (no sandals or flip flops) to protect feet from falling glassware or other lab chemicals that can accidentally drop.

-Put your hair up/secure hair to prevent lab materials from getting into contact with hair and prevent possible confounding lab variables.

-Keep hands out of wells, wear gloves if necessary. To prevent bacteria transfer from your hands or other sources, make sure you do not touch the solutions which embryos are exposed to (prevent possible confounding lab variables).

Results

The fish were observed from their embryo stages to the 96 hour stage where the final observations took place. (See figure 1 for setup and embryo appearance after a day was spent submerged in their respective solutions) Each well contained 10 embryos, so there was a total of 120 fish in our experiment. With 30 of them being in the control group, and leaving the remaining 90 fish to be submerged in Caffeine solutions with differing caffeine concentrations. Thus, the 90 fish in the experimental group would be tested for their survivability rates compared to the control fish groups.

During each observation day pictures were taken of fish in each well under a microscope so their appearances could be noted. The number of dead fish in each well was noted after each day of observation. In addition to observing the amount of surviving fish in each well, we also tested their reflexes by trying to draw out a response by pushing against their tails with a toothpick. All of the eggs developed relatively the same during the first observation days. It is during the second and third day when

the eggs started to differentiate in their development based on the well the eggs were in. The control group embryos hatched in the second day of observation, and had no deaths. The same effect happened in the 0.05 mg/ml Caffeine and 0.25 mg/ml caffeine wells, however in the 1.00 mg/ml wells only one egg hatched and the other eggs had deteriorated and died off. The observations during the second day showed that the eggs had not developed at all in the 1.00 mg/ml wells and the one fish that hatched was severely underdeveloped and its spine had bent into a right angle. It was observed that 100% of the 1.00 g/ml Caffeine fish had died after the first day. Additionally, the control group fish had straight spines, and this observation was also seen in the 0.05 mg/ml Caffeine well, but the fish in the 0.25 mg/ml caffeine well each had deficient spines and had different curvatures to their spine shapes. When their reflexes were tested, the control group fish responded to the toothpick stimulus by swimming away and turning away from the toothpick. The fish in the 0.05 and 0.25 mg/ml Caffeine solution wells did not react as quickly to the toothpick stimulus compared to the control group fish. In the third day and last day of fish observation, all but the 1.00 mg/ml caffeine fish survived in their wells and developed into the next stage of their growth.

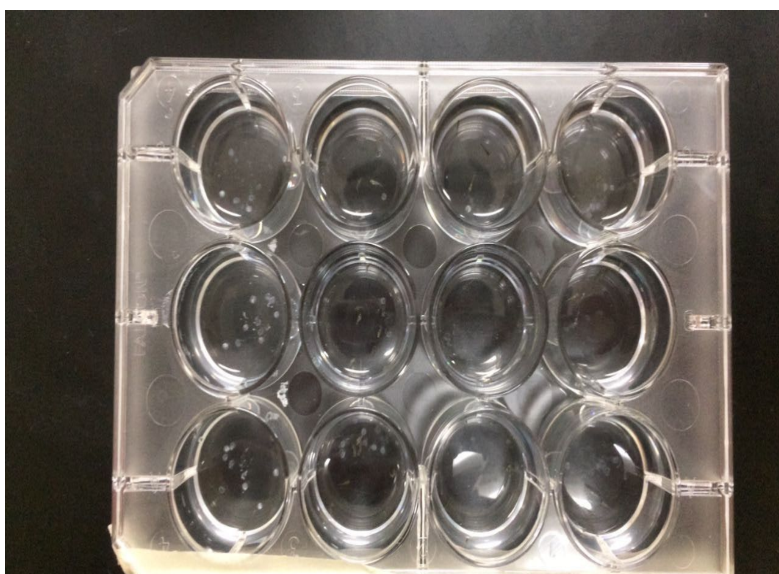
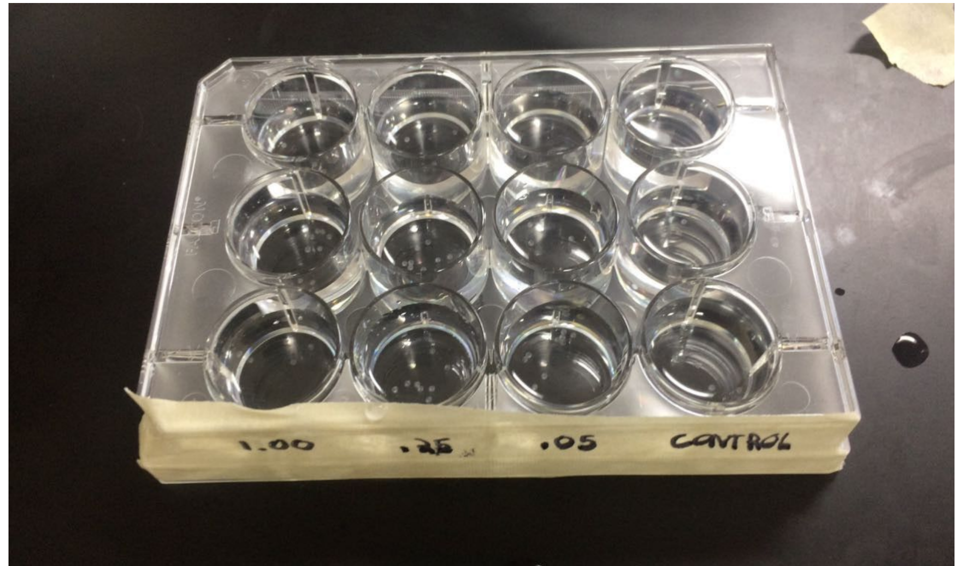


Image 1: Pictured here is the setup of our experimental wells. The column of three wells to the very right contained a solution of 1.00 mg/ml Caffeine, the next column contained a 0.25 mg/ml Caffeine solution, the third contained a 0.05 mg/ml Caffeine solution, and the last column contained the control solution which had no Caffeine added to it.

Image 2: Pictured Below is another perspective of our lab setup for the experimental wells. The units for the caffeine concentrations are in mg/ml


1.00
.25
.05
CONTROL

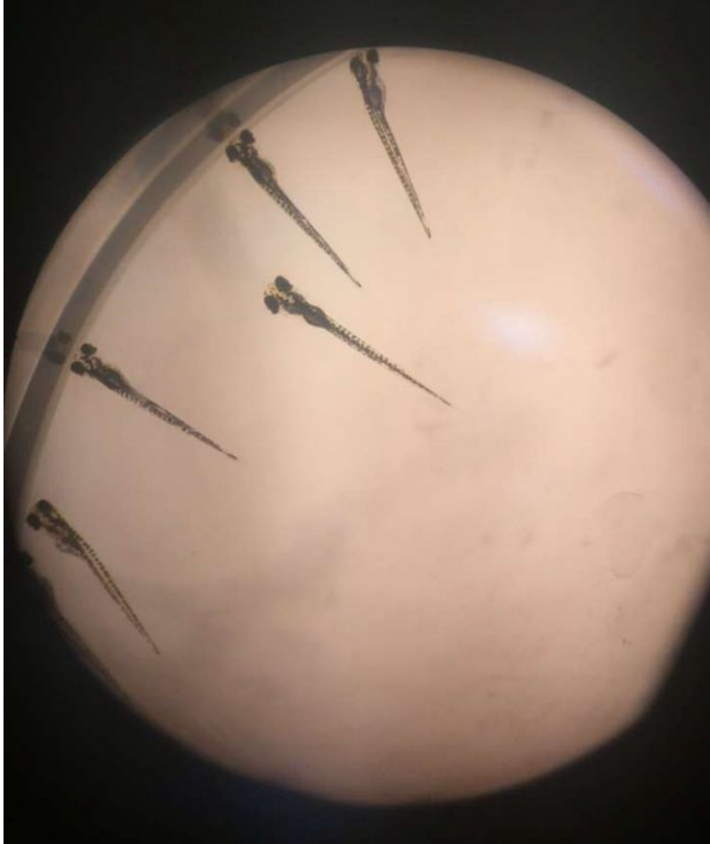


Image 3: Pictured are fish from a control group well under a microscope.
The spines are straight and had no abnormalities.

Image 4: Photo taken of a fish from a 0.25 mg/ml Caffeine well. The fish had a very curved spine and did not respond well to stimuli.

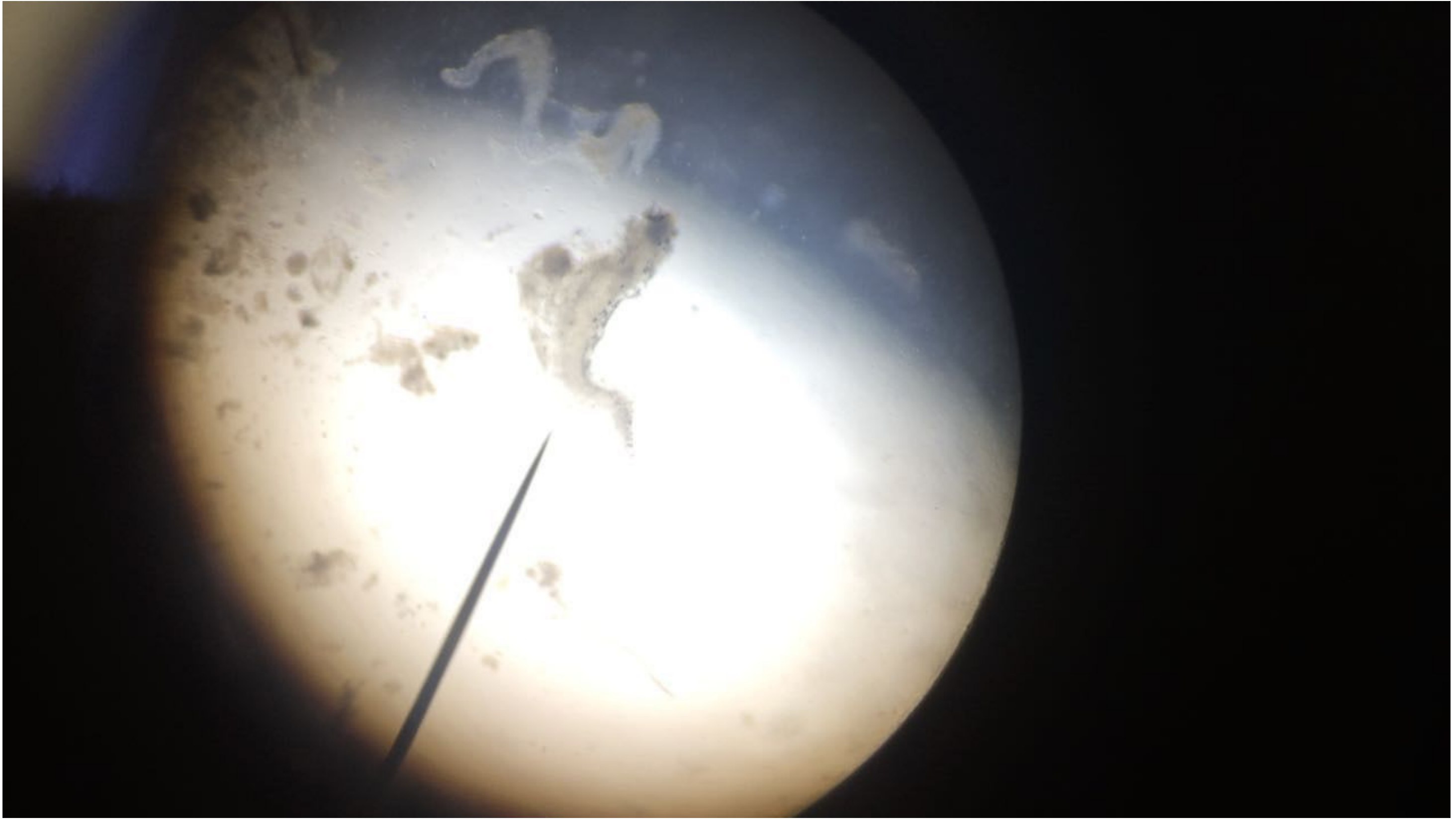


Image 5

Pictured above shows the one fish that hatched in the 1.00 mg/ml Caffeine wells. It was severely deteriorated and had a curved spines as well as other abnormalities and protrusions from its body.

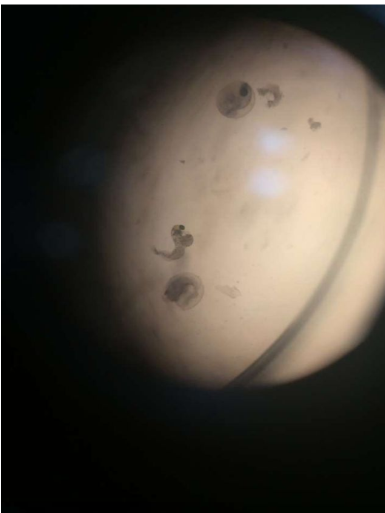


Image 6: An additional picture of a 1.00 mg/ml caffeine well. All of the fish were deceased in their embryo sacs and only one fish fully came out of the embryo stage but still died.

Table 1: Shows the amount of hatched fish in each well in the first day of observation

Amount hatched in the control solution wells	**Amount hatched in the 0.05 mg/ml wells**	**Amount hatched in the 0.25 mg/ml wells**	**Amount hatched in the 1.00 mg/ml wells**
4	3	15	1 (however it was dead)

Table 2: Shows the amount of hatched fish in each well in the second day of observation

Control solution wells	**0.05 mg/ml wells**	**0.25 mg/ml wells**	**1.00 mg/ml wells**
23 Fish Hatched	29 Fish Hatched	29 Fish Hatched	1 (however it was dead)

Table 3: Displays the amount of fish alive in the final day of observation

Fish alive in the control solution wells	**Fish alive in the 0.05 mg/ml solution wells**	**Fish alive in the 0.25 mg/ml solution wells**	**Fish alive in the 1.00 mg/ml solution wells**
30	30	30	None

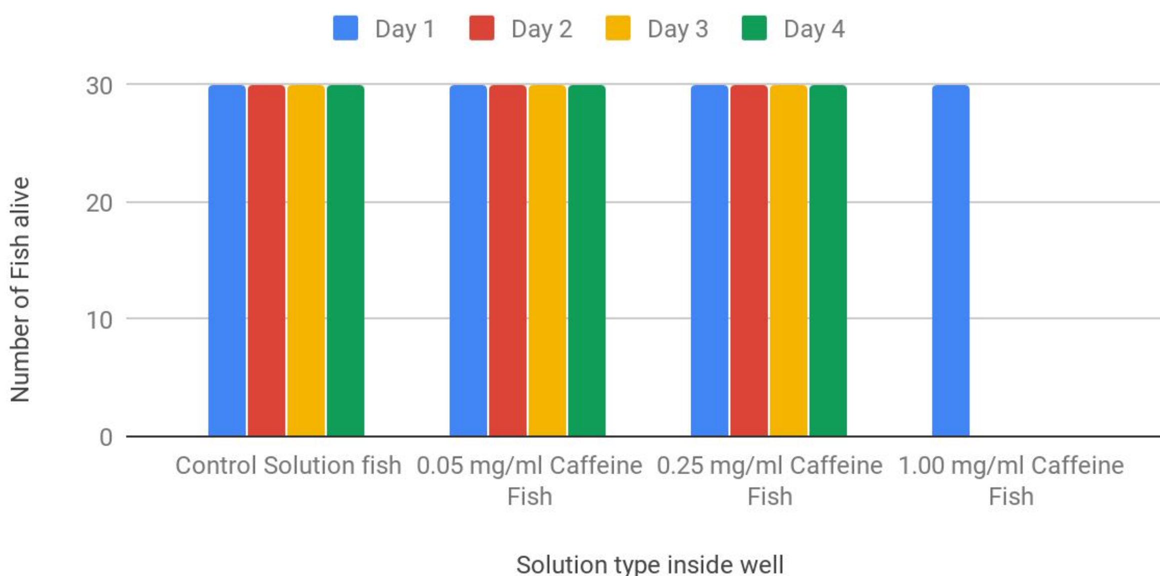


Figure 1: Displays the amount of fish alive in each respective experimental well based on what day it was observed on.

Discussion

It can be reasonably inferred that caffeine indeed had a profound effect on the survivability and wellness rates on the developing zebrafish. The initial statement was that as caffeine concentration increased the mortality rates of the developing fish would also increase, as well as a general decrease in function of their developing bodies. This statement can be supported by the observation of the control solution fish, which had straight spines (See Image 3). There was a noticeable difference between the spine structure and shape between control fish and 0.25 mg/ml fish (See image 4), in which the fish in a 0.25 mg/ml caffeine environment had abnormal and bent spine shapes. The zebrafish also differed in another area, reactability to stimuli. When a toothpick or other obstructive aggressor was pushed up against a control solution fish, the fish fled and reacted quickly as if it was a reflex arc response to a threat. However, as the caffeine concentrations increased within the experimental fish groups, their reactibility to such a stimulus decreased significantly. For instance, the 0.05 mg/ml caffeine solution fish reacted quick, but slower than the control solution fish, to the obstructive stimuli, they additionally were very "jittery" and made more random movements than the control solution fish. However, the 0.25 mg/ml fish did not react well at all to stimuli compared to the two preceding fish groups. This indicates that caffeine indeed had an effect on the development process of eggs. Since caffeine targets the nervous system and increases excitability, it would clash with our data. However, overstimulation and overuse of

caffeine can lead to degrading effects, which explain why the 0.25 mg/ml caffeine solution fish did not respond at all compared to the 0.05 mg/ml caffeine fish, which darted around and was more active because of its relatively low caffeine concentration environment.

Moreover, caffeine has previously been observed to target skeletal muscle as well. In a fully developed organism, negative caffeine effects on bone metabolism would not lead to the intense detrimental outcomes seen in this lab. But in a developing embryo, which requires a neutral environment to ensure that the development can be streamlined with no complications, a decrease in bone metabolism and an increase in developmental process speeds would be disastrous to the growth of the embryo. These effects are echoed by the emergence of the fish from their eggs in the 0.25 mg/ml and their cured spinal structure. This connection shows a direct relationship between the addition of caffeine and its effects on the skeletal system of developing embryos. The 1.00 mg/ml fish were the fish to be the most affected by caffeine complications. 100% of the 30 fish in the 1.00 mg/ml wells had died after the first observation day, and all but one fish in those wells stayed in the eggs. The one fish that had emerged had the same abnormal spine shape as the 0.25 mg/ml fish (See image 5). A little more than 80% of the fish in the 0.25 mg/ml wells had abnormal spine shapes. Since all the wells had similar survival rates except the 1.00 mg/ml caffeine wells, standard deviation could not be calculated.

This could also prove how caffeine does boost fat metabolism and uses more fatty acids as energy rather than muscle glycogen (Stock, Mehl, Buggy, et al, 2002), because further research of zebrafish embryo development determined that “fatty acids [are] required for normal growth and development [of the zebrafish]” (The Zebrafish Husbandry Association, n.d.). Thus, it can be inferred that the fish exposed to the caffeine in higher amounts could have had a greater impact on fat metabolism, leading to more serious growth and development problems as the concentration increased. This would also surely explain the results within the lower concentrations, where we see defects in growth, but the fish still survived and hatched. Perhaps the embryos of the zebrafish were using too much of the fatty acids as energy (because caffeine would cause the burning of fatty acids and inhibit the use of muscle glycogen as energy), preventing the embryo from using the lipids for growth and development instead, and ultimately leading to either the demise of the embryos (seen in the caffeine 1.00 mg/ml solution) or the defects in growth (seen in both the caffeine .25mg/ml solution and the 0.05 mg/ml solution). Either way, it is a known fact that fat is needed in the development of the embryos, and since the embryos were defects or died in the caffeine solutions, it is safe to assume, from the results of this lab, that fat metabolism certainly plays a role in embryonic development. Fat metabolism involvement in embryonic development is also profoundly visible within humans. Pregnant females are recommended to take no more than 200mg of caffeine each day, as it increases the chance of miscarriages (Davis, 2014), perhaps 200mg of caffeine is the limit to how much a human embryo can take before there are not enough lipids present for growth and development (because it is being wrongfully used as energy in substitute of muscle glycogen), causing miscarriage (the fetus/embryo will not grow and will die). Either way, the impacts of caffeine and fat metabolism is proven both ways (that it does impact fat metabolism or that it doesn’t), but the results of this lab helps conclude that caffeine does play a role in increasing fat metabolism.

This empirical evidence further supports the theory that caffeine has a detrimental effect on embryonic development, effects that will stay with the fish forever and that cannot be remedied.

Obviously there were limitations to the experiment, as the growing and developing fish could not be watched all day. The fish were instead observed each day after they were taken out of an incubator. There were some errors in the experiment as well, as some of the wells may not have had 10 fish each because of errors that occured while the solutions were being replaced each observation day. In addition, the caffeine solutions that were used were recycled from other groups testing caffeine because of the limited caffeine amount that was available. This may have affected the solution's caffeine concentration and the final observations that were made on the developing zebrafish. Overall, the lab yielded results that helped prove the significant impact caffeine has on bone and fat metabolism on embryos, and supported the hypothesis in that the higher caffeine concentrations would result in a higher number of fish with genetic defects and abnormal function.

References

Caffeine: How much is too much? (2017, March 08). Retrieved from https://www.mayoclinic.org/healthy-lifestyle/nutrition-and-healthy-eating/in-depth/caffeine/art-20045678.

Juliano, L. M., Huntley, E. D., Harrell, P. T., & Westerman, A. T. (2012, February 15). Development of the Caffeine Withdrawal Symptom Questionnaire: Caffeine withdrawal symptoms cluster into 7 factors. Retrieved from https://www.sciencedirect.com/science/article/pii/S0376871612000269#bib0120.

Bolton, S., Ph.D, & Null, G., M.S. (1981). *Caffeine Psychological Effects, Use and Abuse*[PDF]. Retrieved from http://orthomolecular.org/library/jom/1981/pdf/1981-v10n03-p202.pdf.

Huang TH, Yang RS, Hsieh SS, Liu, SH. (2002, January 30). *Effects of caffeine and exercise on the development of bone: a densitometric and histomorphometric study in young Wistar rats*. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11792600.

Dubuc, B. *Caffeine*. Retrieved from http://thebrain.mcgill.ca/flash/i/i_03/i_03_m/i_03_m_par/i_03_m_par_cafeine.html.

Sinicki, A. (2014, July 23). *How Caffeine Affects Neurotransmitters and Profoundly Changes Your Brain*. Retrieved from http://www.thebioneer.com/caffeine-affects-neurotransmitters-profoundly-changes-brain.

Gavin, M. (2014, September). *Caffeine*. Retrieved from https://kidshealth.org/en/teens/caffeine.html?WT.ac=t-ra.

Harvard Medical School. (2015, July). *Can Caffeine Really Stunt Your Growth?* Retrieved from https://www.health.harvard.edu/staying-healthy/can-coffee-really-stunt-your-growth.

Saville, J. *Coffee and Kidney Disease: Is It Safe?*. Retrieved from https://www.kidney.org/newsletter/coffee-and-kidney-disease.

Stock, H.S., Mehl, K.A., Buggy, J., Davis, M.J., Zhao, Z, & Hand, G.A. (2002, October 21). *Central Nervous Effects of Caffeine and Adenosine on Fatigue*. Retrieved from https://pdfs.semanticscholar.org/200e/d587d048948a5d57beb768021432ec2c39ca.pdf?_ga=2.55607659.1163895866.1545354527-2042456914.1545354527.

Davis, S. (2014, June 4). *Does Drinking Caffeine Increase Your Risk Of Miscarriage?* Retrieved from https://www.webmd.com/baby/features/does-caffeine-raise-risk-of-miscarriage.

Santoriello, C. & Zon, L.I. (2012, July 2) *Hooked! Modeling Human Disease in Zebrafish*. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3386812/.

The Zebrafish Husbandry Association. *Zebrafish Basics*. Retrieved from http://zhaonline.org/zebrafish-basics.html.