Effects of Caffeine on the Development of Zebrafish

By: Ahnna Faust, Hailee Fisher, Felicity Giampietro

Divine Savior Holy Angels High School

Abstract:

The purpose of this experiment was to determine the effects of caffeine on the embryonic development of zebrafish. To do so, fertilized zebrafish eggs were placed in solutions of varying concentrations of caffeine (0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL) as well as a control solution (absent of caffeine). Over a three-day testing period post-fertilization, the number of living zebrafish was recorded along with the number of hatched eggs for each solution. Physical developmental changes such as curved spine, enlarged yolk sac, stunted growth and movement behavior were all examined on the last day of the testing period (72 hours post-fertilization). The most notable consequence of the solution with the highest caffeine concentration was the 87% death rate of zebrafish after 72 hours post-fertilization compared to the 0% death rate of the control group 72 hours post-fertilization. The medium caffeine concentration of 0.25 mg/mL also displayed negative effects in the physical abnormalities of the zebrafish such as curved spine, stunted growth, and enlarged yolk sac. Zebrafish were chosen to use in this experiment because of their genetic similarities with humans. Therefore, the damage done by the higher doses of caffeine to the zebrafish embryos is telling of the damage caffeine can do to human fetuses as well— heavy and consistent intake can cut off blood flow in the placenta. This can be a detriment to the fetus' development, just as it was to the zebrafish embryos- stunting growth and increasing the potential for a miscarriage.

Purpose Statement:

What is the effect of caffeine on embryonic zebrafish development?

Background Research:

Caffeine is a chemical that stimulates the central nervous system and is found in a wide range of commodities - both natural and manufactured. Coffee beans, tea leaves, as well as kola nuts and chocolate contain traces of the chemical and are in turn used to create energy drinks, soft-drinks, tea, and coffee (Bond). Caffeine is also present in a range of pharmaceutical drugs such as acetaminophen and aspirin. The majority of caffeine that is consumed, however, comes from the aforementioned beverages of coffee, tea, etc. (Mitchell). One study looked at data from the most recent cycle of the National Health and Nutrition Examination Survey in 2012, which showed that "the largest contributors to dietary caffeine were coffee (64%), tea (18%), and caffeinated sodas (15%). Energy drinks accounted for 2% of caffeine (2.7 mg/day) and foods contributed 2.0 mg/day or 1.5% of total caffeine" (Drewnowski). Thus, the main source of caffeine intake proved to be coffee. Caffeine is present in a number of different products - both those naturally occurring and those that are manufactured.

Because of its presence in a multitude of products, caffeine is easily accessible and is often called into question as to whether or not it has adverse effects on human health - especially the health of pregnant women. According to one study done regarding the demographics of caffeine-intake, " 70-95%' of pregnant women consume caffeine from various sources each day, with an average caffeine intake during pregnancy of 99- 185 mg/day" and further still, "a range of 5.7 to 29.7 of pregnant women are heavy consumers of caffeine (2300 mg/day) during pregnancy" (Hinds). These statistics exemplify the large prevalence caffeine has among pregnant women and society. The question, then, begs what effects the intake of caffeine has on the developing fetus.

When caffeine is consumed by a pregnant woman, it crosses the placenta into the amniotic fluid and the fetus. However, "Neither the fetus nor the placenta can metabolize caffeine because they lack the necessary enzymes, so the fetus is exposed to caffeine and its metabolites in proportion to maternal exposure for a prolonged period of intra-uterine life" (Grosso). This ultimately indicates that while the mother's body works to eliminate caffeine from the body, the fetus remains exposed to the chemical. During the third trimester, the rate of a mother's body being able to break down caffeine decreases, causing even greater levels of the chemical to remain in the bloodstream. This means that more caffeine is crossing the placenta and reaching the fetus - who is still unable to process it efficiently. Various health complications have been linked to exposure to extreme amounts of caffeine by the developing baby including premature labor/delivery, abnormally low birthweight, and congenital deformities. According to one study, "Total caffeine consumption of >300 mg daily during pregnancy has been associated with reduced birth weight" (Hinds). The study also concluded that, "caffeine has a vasoconstrictive effect on placental intervillous blood flow" meaning it ultimately narrows blood vessels and restricts blood flow. This is a detrimental effect of the chemical, as it inhibits the proper development of the baby in utero. Another study of 711 women determined, "the adjusted odds ratios of spontaneous abortion by caffeine consumption to be 2.20 (141-280 mg/day), 4.81

(281-420 mg/day), and 15.43 (>421 mg/day) (Hinds). This study in particular demonstrated that high caffeine consumption is linked to - and in fact, increases- the potential for miscarriage.

Caffeine is present in a number of naturally occurring products, drinks, and foods. It is consumed frequently and in most cases daily by the majority of society, including pregnant women. Caffeine can have numerous negative effects on the developing baby in-utero including congenital deformities and in some cases, miscarriage.

Null Hypothesis:

An increase in the amount of caffeine will have no effect on the physical development or survival of zebrafish.

Alternative Hypothesis:

An increase in the amount of caffeine will negatively affect the physical development and survival of zebrafish.

Materials:

12-well well plate
96 fertilized zebrafish embryos
“Instant ocean” salt water solution, 6.0 mL
0.05 mg/mL caffeine solution, 6.0 mL
0.25 mg.mL caffeine solution, 6.0 mL
1.0 mg/mL caffeine solution, 6.0mL
Small pipette
Large pipette
Methylene blue dye
Incubator
Dissecting microscope
Beaker

Methods:

Day 1– Set Up

1. 8 fertilized zebrafish eggs were placed in each of the 12 wells in the well plate as shown in Figure 1.
2. 2.0 mL of "instant ocean" salt water solution was added to the wells in column1 of the tray (*see figure 1*)
3. 2.0 mL low concentration (0.05 mg/mL) of caffeine was added to three wells of the second column (*see figure 1*)
4. 2.0 mL medium concentration (0.25 mg/mL) of caffeine was added to three wells of the third column in the tray (*see figure 1*)
5. 2.0 mL high concentration (1.0 mg/mL) of caffeine was added to three wells of the fourth column in the tray (*see figure 1*)
6. The pipette was dipped in the methylene blue dye and added to the first column of 3 wells— the control solution.
7. The pipette was dipped in the methylene blue solution and added to every well in columns 2-4.
8. Entire well plate was placed in an incubator at 28.5 degrees C overnight.

Days 2-3– Data Observations

1. Dissecting microscope was used to observe the zebrafish in each well solution.
2. Quantitative data from observation was recorded in the data table (dead or alive / # of eggs hatched / physical development of fish*)
    a. *only record physical developments on day 3 in addition to other observations
3. Dead embryos were removed from each well after making initial observations with the dissecting microscope.
4. Environmental factor solutions were removed from each well plate using a pipette by tilting the plate so that the embryos settled to one side and then removed the liquid from the top.
5. Steps 2-8 were repeated from Day 1.

Data:

Data Table A. Number of Hatched and Living Zebrafish Embryos

Treatment	Well #	# of starting fish	48 hours post fertilization		72 hours post fertilization	
			# hatched	# live	#hatched	# live
Instant Ocean (Control)	A1	8	0	8	8	8
	B1	8	0	8	5	8
	C1	8	0	8	7	8
Caffeine 0.05 mg/mL	A2	8	0	8	6	6
	B2	8	0	8	5	6
	C2	8	0	8	5	8
Caffeine 0.25 mg/mL	A3	8	0	8	6	6
	B3	8	2	8	8	7
	C3	8	1	8	6	6
Caffeine 1.0 mg/mL	A4	8	0	8	0	0
	B4	8	0	5	0	3
	C4	8	0	6	0	0

Data Table B. Qualitative Observations of Zebrafish Development in Experimental/Control Groups 72 Hours Post Fertilization

Control/Experimental Groups	Observations
Control Group	no stunted growth or curved spine, some excited movement, normal yolk sacks
Low Concentration (0.05 mg/mL)	more active movement, slightly larger yolk sack, no curved spine or stunted growth
Medium Concentration (o.25 mg/mL)	enlarged or exploded yolk sack, clear curved spine and stunted growth, circular movement or twitching in place but normal swimming

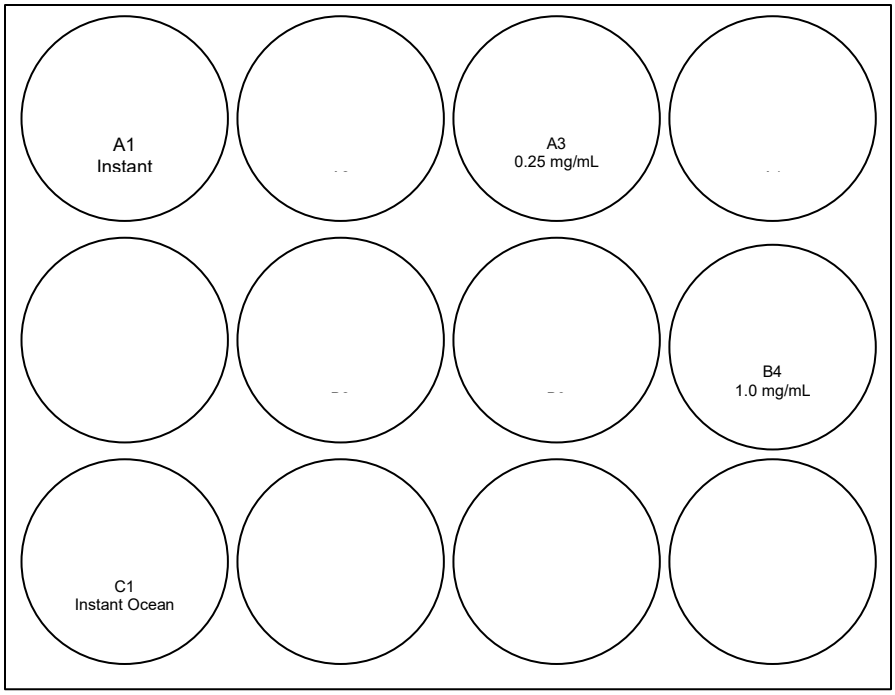
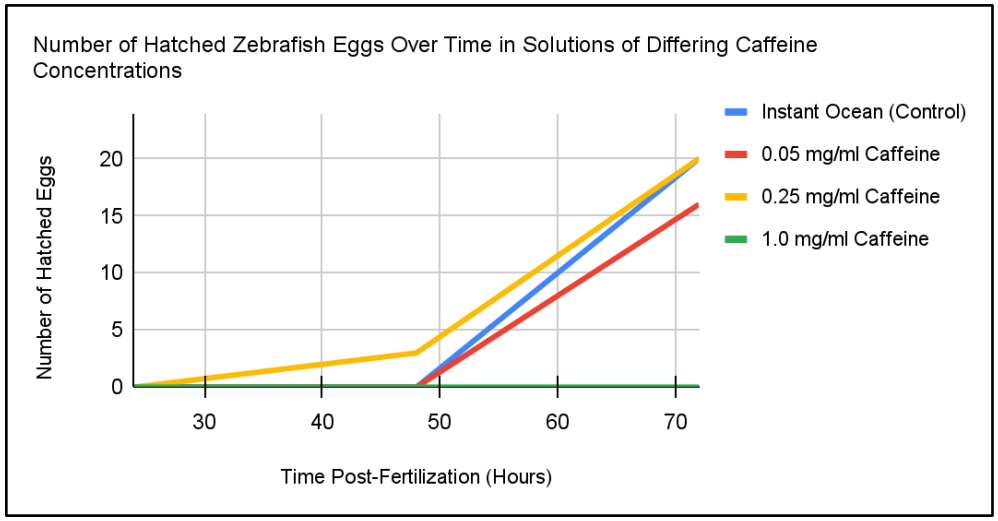
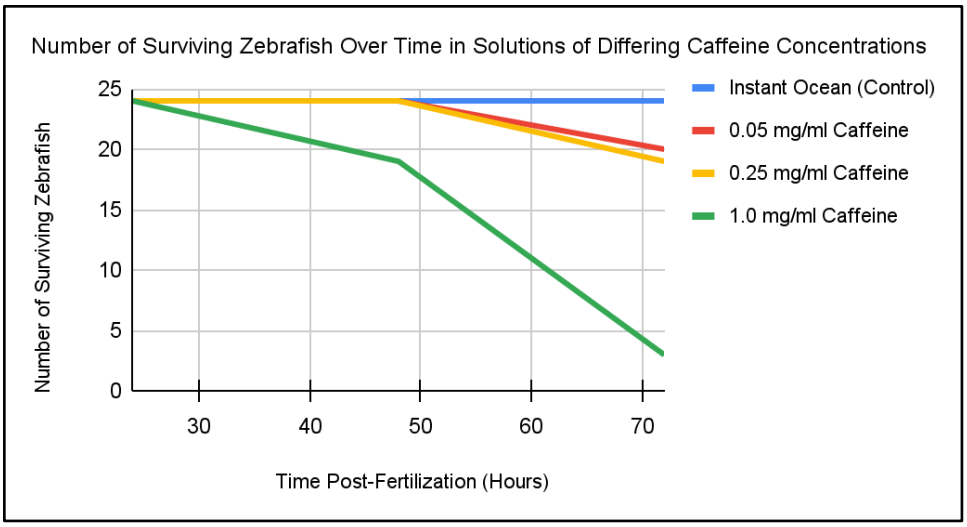


Figure 1

Graphs:





Statistical Significance:

Results Table A. Statistical Analysis of Number of Fish Alive

	Mean	Standard Deviation	P- Value	Statistically Significant
Control	8.00	0.00		
Low Concentration	6.67	1.15	0.1161	No
Medium Concentration	6.33	0.58	0.0075	Very
High Concentration	1.00	1.73	0.0022	Very

The results between the low concentration of caffeine and low concentration are not statistically significant because the p-value is over 0.05 at 0.1161. Because it's not a statistically significant difference in data, chance cannot be ruled out as a cause for the difference in data, and it then cannot be concluded that caffeine is the reason for the lower number of fish alive after 72 hpf. However, the medium and high concentrations are both very statistically significant compared to the control group because the p-values are .0075 and 0.0022 respectively. Therefore, it can be said that the caffeine, not chance or any other lurking variable or factors, caused the lower number of alive zebrafish after 72 hpf.

Results:

During the initial placement of the zebrafish into their respective wells and various levels of caffeine, they all were unhatched, healthy, and alive. Each of the 8 zebra fish in their respective wells was alive 24 hpf, moving within their sacks and displaying normal developmental growth as shown in Figure 2. After 72 hpf, there were some differences between each of the various concentrations of caffeine. The control group in a caffeine-free environment that best replicates the zebrafish' natural habitat had all 24 fish alive, and 21/24 of the total fish in the control group hatched. Zebrafish in this experimental group displayed normal growth patterns without any abnormalities and displayed normal and frequent movement throughout each of the wells, as shown in Figure 3. There were no enlarged yolk sacs, stunted growth, or curved spines. The experimental group with low concentration of caffeine, 0.05 mg/mL showed minimal changes compared to the control group. Of the 24 total fish from the start of the experiment, 20 of them survived 72 hpf. A smaller number of them hatched compared to the control group; the low concentration hatched 16 compared to the 21 of the control group. However, there were not very many developmental differences between the two groups aside from a slightly enlarged yolk sac compared to normal. The alive and hatched fish in the low concentration group exhibited more excited and frequent movement compared to the control group. The greatest developmental abnormalities with alive fish occurred in the 0.25 mg/mL medium concentration of caffeine. Even less than the low concentration, only 19 of the 24 zebrafish were alive at 72 hpf, yet more of them, 20 out of the 24, hatched than the low concentration group. Figure 4 shows the curved spines, enlarged yolk sacs, and stunted growth that the zebrafish in the medium concentration who were still alive 72 hpf experienced. Further, many of them had excited or quick movements in a circle or twitched repeatedly instead of showing normal swimming motions. There was a drastic change between the 1.0 mg/mL high concentration of caffeine and the other three experiment groups because 0 of the fish hatched and 21 of them died by 72 hpf. Their opaque egg sacs and indistinguishable physical features are shown in Figure 5. The greatest difference in the number of zebrafish in the high concentration alive occurred between the 48 hpf and 72 hpf because the number of alive fish dropped from 19 to 0.

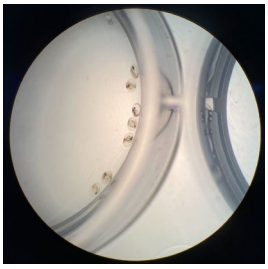


Figure 2.
This picture displays healthy unhatched zebrafish eggs from the control group. It was taken during the first data collection day, approximately 48 hours post fertilization.

Figure 3.
Nearly 72 hours post fertilization, this picture shows healthy hatched zebrafish from the control group. They showed normal movement and developmental growth.

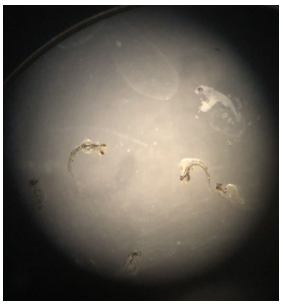


Figure 4.
This picture shows hatched zebrafish from the 0.25 mg/ml medium concentration group about 72 hpf. The zebrafish in this experimental group had limited movement due to stunted growth and curved spines.

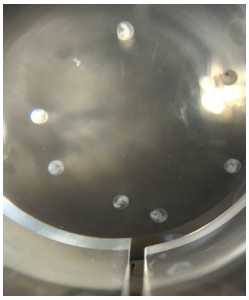


Figure 5.
After 72 hpf, all of the zebrafish in the 1.0 mg/ml high concentration caffeine group remained unhatched and dead, as shown in the photo above. This large change occurred between the 48 and 72 hpf observational periods.

Discussion

An increase in the concentration of caffeine does impact the embryonic development of zebrafish in terms of physical abnormalities including curved spine, enlarged yolk sac, stunted growth, movement behavior, and quickened hatching rate. The data sufficient to support this claim is observed in the growth and behavioral abnormalities exhibited in the medium and high concentrations compared to the control group after 72 hours post-fertilization. Qualitatively, it was found that the yolk sacs of the zebrafish in the medium concentration (0.25 mg/mL) after 72 hours were noticeably bigger than those of the control group - to the extent that one of the zebrafish died from a ruptured sac. In addition to the enlarged yolk sac, the zebrafish in the medium concentration were also more active in their movement compared to the control group as they twitched and spun in circles. Lastly, the zebrafish in the medium concentration also developed visibly curved spines and were noticeably smaller in size than those of the control group. In regards to the high concentration (1.0 mg/mL), none of the zebrafish hatched and no movement was seen or noted. Quantitatively, the numbers of surviving fish from both the medium and high concentrations were very statistically significant. After 72 hpf, only 19 fish were living in the medium concentration, compared to the 24 fish that were alive in the control solution. The effects of the caffeine became most noticeable, however, when it came to the high concentration, in which only 3/24 fish remained living after 72 hpf. This meant that there was an 87% (21/24) death rate for zebrafish in the highest concentration of solution versus a 0% (0/24) death rate for zebrafish in the control group.

At the medium concentration (0.25 mg/mL), negative issues were first noticed. The average 8-ounce cup of coffee has a caffeine concentration of roughly 0.40 mg/mL, which has a concentration valued between that of the medium and high used in this lab. As coffee was proven the number one source of intake for caffeine, this number became important. Had another solution containing 0.40 mg/mL caffeine been tested during this experiment, it would be expected to exhibit results similar to those of the medium and high concentrations - or somewhere in between. Because negative issues were first noted at the medium concentration, it can be concluded that coffee- with its concentration being greater than that of the medium experimental group - could potentially have the same adverse effects on the development of a human fetus (as a similar concentration had on the zebrafish) when consumed in large amounts by the mother.

The abnormally small size of the zebrafish was first seen in the medium concentration (0.25 mg/mL). This effect is similar to the effects that high amounts of caffeine had on pregnant mothers whose babies were reported to have low-birthweight. Both of these ideas are biologically plausible. High caffeine intake can restrict blood flow in the placenta, and any reduction in blood flow in the placenta is directly linked to low-birthweight/decreased fetal growth.

The low number of surviving zebrafish after 72 hpf in the high concentration is an especially important number to examine when discussing miscarriage potential due to high amounts of caffeine intake. The high concentration showed the greatest number of deceased

zebrafish: 21/24 (87%). As would be expected, a higher concentration of caffeine would restrict blood flow in the placenta even more so than a medium concentration, leading to perinatal morbidity. Thus, the high concentration experimental group and the overwhelming number of deceased zebrafish within the group reflects the higher potential for miscarriage that high levels of caffeine creates.

A possible source of error in this experiment could have been that when step one of the procedure was being executed, one of the eggs added to a well was not fertilized. The eggs were not examined very closely upon deposit into each of the wells. This error could have led to an artificially low value of living zebrafish for any of the experimental groups. Further, upon removal of the dead zebrafish and replacement of the solutions in steps 3 and 4, the pipette could have made harsh contact with a living egg, causing the zebrafish to die prematurely. The eggs were not examined after the solution was added to the wells in step 4, and it is unclear whether this could have played a part in the number of surviving zebrafish for any of the experimental groups.

One important limitation of the lab was the length of time in which it was conducted. Had there been a greater amount of time to observe the continued growth and development of the zebrafish, better observations could have been recorded regarding their physical development, leading to more conclusive evidence. More time would have allowed for there to be a greater distinction between the effects of the low concentration and medium concentration solutions on the development of the zebrafish.

Works Cited

Bond, Susie. “Caffeinated culture: Looking at the good and bad of caffeine consumption” *Reno Gazette - Journal,* 12 April, 2021. *U.S. Newsstream*, https://www.proquest.com/newspapers/caffeinated-culture/docview/2511065842/se-2?accountid=8461.

Mitchell, D. C., Knight, C. A., Hockenberry, J., Teplansky, R., & Hartman, T. J. (2013, November 1). *Beverage caffeine intakes in the U.S.* Food and Chemical Toxicology. Retrieved December 1, 2021, from https://www.sciencedirect.com/science/article/pii/S0278691513007175#b0045.

Drewnowski, A., & Rehm, C. D. (2016, March 10). *Sources of caffeine in diets of US children and adults: Trends by beverage type and purchase location*. Nutrients. Retrieved December 1, 2021, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4808882/#B1-nutrients-08-00154.

Tanya S. Hinds, R.D, William L. West, Ph.D, Enid M. Knight, Ph.D., L.N, Barbara F. Harland, Ph.D., R.D., L.D., L.N, The Effect of Caffeine on Pregnancy Outcome Variables, *Nutrition Reviews*, Volume 54, Issue 7, July 1996, Pages 203–207, https://doi.org/10.1111/j.1753-4887.1996.tb03933.x

Grosso, L. M., Triche, E., Benowitz, N. L., & Bracken, M. B. (2008, March). *Prenatal caffeine assessment: Fetal and maternal biomarkers or self-reported intake?* Annals of epidemiology. Retrieved December 3, 2021, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2275917/.

Lamy, Sandrine, et al. "Caffeine Use during Pregnancy: Prevalence of Use and Newborn Consequences in a Cohort of French Pregnant Women." European Archives of Psychiatry & Clinical Neuroscience, vol. 271, no. 5, Aug. 2021, pp. 941–950. EBSCOhost, doi:10.1007/s00406-020-01105-2.