Effect of Caffeine on Zebrafish Development

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Abstract:

The purpose of conducting the Zebrafish Lab Report is to use a real life model to show how caffeine affects pregnant women and their developing fetus. We placed zebrafish in three different concentrations of caffeine: .05 mg/ml, .25 mg/ml, and 1.0 mg/ml, and we recorded how many hatched and how many lived 24, 48, and 96 hours post fertilization. After conducting the experiment, we found that the increased death rates and decreased hatching rates corresponded with higher caffeine concentrations. In addition, we discovered that the .05 mg/ml group and the .25 mg/ml group produced similar results, but there was a clear distinction between the caffeinated groups and the control group. The least amount of deaths and greatest amount of hatched zebrafish were found in this control group (Instant Ocean Solution), which best represents the habitat zebrafish normally live in. After experimenting with this model, we came to the conclusion that it is not safe or healthy for pregnant women to consume a large amount of caffeine. It is best for pregnant women not to consume any at all, but if they do, they should not intake more than recommended by a health professional, as it could harm the baby physically and slow or prevent it's development. Any amount of caffeine could negatively affect a baby's health, physical characteristics, and process of being born, but as the concentration increases, the likelihood of side effects does as well.

Purpose Statement/Objective:

What is the effect of caffeine on zebrafish development?

Background research:

Caffeine can be found naturally in tea leaves, cocoa beans and coffee beans, which are the plants used to make tea, chocolate and coffee. It is also found in a majority of energy drinks and other sodas such as Coke, Pepsi, Mountain Dew and Dr. Pepper. One twelve ounce can of soda is equivalent to 37 milligrams (mg) of caffeine. For adults, caffeine intake is highest in coffee, and many brands of coffee add extra caffeine so that as much as 360 mg of caffeine can be in a single cup (American Pregnancy Association). The most common sources of intake for young children are soda, candy bars, mints and gum (Cheriyedath, Susha).

Approximately 90% of Americans consume caffeine in some form, and it is labeled as "America's most popular drug" (*America's Most Popular Drug*). What is considered a 'safe' amount of caffeine for adults is 400 mg. Most coffee drinkers drink two to three cups per day, but any amount can cause side effects. Once caffeine is introduced into one's system, it can be hard to function correctly without it since the body becomes used to having a certain amount of caffeine running through the blood vessels. If this amount is reduced, it shocks the body and can put one's health at risk. This is a problem for our society because so many people are 'addicted' to caffeine and feel as though they cannot live without it (Mayo Clinic).

Caffeine has its benefits, such as reducing drowsiness, helping with sleep deprivation, providing an energy boost and heightening awareness levels, but there is a lengthy list of side effects that can not be overlooked. Caffeine is a mild stimulant of the central nervous system, and it is a psychoactive drug that can alter moods and behavior. Prolonged caffeine consumption can decrease the body's ability to absorb nutrients, it can disturb sleep, worsen stomach problems, trigger an irregular heartbeat, promote fatigue, and it can cause panic attacks and

dehydration. Withdrawal symptoms include headaches, fatigue, muscle pain, sweating, anxiety and more (*America's Most Popular Drug*). According to a US news report, caffeine consumption can cause “jitteriness, nervousness, upset stomach, problems sleeping and concentrating.” As caffeine intake increases, side effects expand to include nausea, vomiting, diarrhea and frequent urination, restlessness, an increased heart rate and the possibility of an irregular heart rate. Overdosing can cause seizures and cardiac arrest along with heightening nerve or heart disorders (US News Health). It is for these reasons that caffeine should be consumed in moderation.

It may appear as though adults consume more caffeine than children, but as it is found in a vast variety of food and drinks, research has discovered that 73% of children consume caffeine on a daily basis. A 2015 study published by the Journal of Human Lactation found surprising rates of coffee consumption among toddlers aged 1 to 2 years old. 2.5% of one year olds were drinking coffee, and over 15% were consuming coffee by the age of two (US News Health). In 2010, the Journal of Pediatrics published a study in which 75% of children participating consumed caffeine daily, and the more they consumed the more awake they were (Cheriyedath, Susha). These statistics are concerning as caffeine has been found to increase blood pressure and slow down one’s heart rate along with all the other side effects previously mentioned. Due to these reasons, children younger than twelve should avoid caffeine. The smaller the person, the less caffeine needed to pose these health concerns. According to the American Association of Poison Control Centers, approximately 1,200 cases of caffeine toxicity in children under age six are reported each year (Cheriyedath, Susha).

Caffeine doesn't just affect children and adults. It can also harm a developing fetus if pregnant women are not cautious. Caffeine crosses the placental barrier to the unborn child, and in nursing mothers, it is excreted through breast milk (*America's Most Popular Drug*). Caffeine consumption while pregnant can lead to premature labor and birth defects. Women who consume 200 mg or more of caffeine daily are twice as likely to have a miscarriage as women who do not take in any caffeine. According to a 2015 study, the risk of miscarriage rose by 19% for every increase of 150 mg per day of caffeine and by 8% for every increase of two cups of coffee per day. In another study, there was no increased risk in women who drank between 200 and 350 mg coffee daily (American Pregnancy Association). An examination of studies published between 1975 and 1999 reported minimal effects of caffeine ingestion during pregnancy on neurodevelopment and behavior in infants and children, but there have been several cases of caffeine withdrawal reported in infants whose mothers consumed more than 800 mg of caffeine per day. Pregnant women are advised to not consume more than 200 mg per day, and mothers who breastfeed should not drink more than two cups a day (Morgan, Sara).

Null Hypothesis: An increase in the caffeine concentration will have no effect on the zebrafish development.

Alternative Hypothesis: An increase in the caffeine concentration will slow the process of zebrafish development and increase the number of zebrafish deaths.

Materials:

64 zebrafish eggs
24-well plate

Instant ocean solution
3 caffeine concentrations: .05 mg/ml, .25 mg/ml, 1.0 mg/ml
Methylene blue solution
1 large pipette
1 small pipette
Dissecting microscope
Beaker
Incubator

Methods:
Day 1

1. Place 4 zebrafish eggs into 16 wells using a small pipette
2. Add 1 ml of instant ocean solution to the four wells in column 1 using a large pipette
3. Add 1 ml of low concentration caffeine solution (.05 mg/ml) to the four wells in column 2 using a large pipette
4. Add 1 ml of medium concentration caffeine solution (.25 mg/ml) to the four wells in column 3 using a large pipette
5. Add 1 ml of high concentration caffeine solution (1.0 mg/ml) to the four wells in column 4 using a large pipette
6. Dip a large pipette in methylene blue solution and then into each well, starting with control group
7. Put the 24-well plate in incubator at 28.5 degrees celsius overnight

Day 2-4

1. Observe zebrafish through dissecting microscope
2. Days 2-4 gather quantitative data (number of alive zebrafish and number of hatched zebrafish) and fill in "Number of Living and Hatched Zebrafish Embryos" data table. On day 4 only, make note of physical development of zebrafish in "Day 4 physical development chart"
3. Remove dead embryos from the well plate using a small pipette. Release them into an empty beaker. Start with control group, then low to high concentration
4. Remove environmental factor solutions from each well using a large pipette. Release into the beaker.
5. Repeat steps 2-7 from day one

Number of Living and Hatched Zebrafish Embryos

Treatment	Well #	# of	24 hours post	48 hours post	96* hours post

		starting fish	fertilization		fertilization		fertilization	
—	—	—	# hatched	# live	# hatched	# live	# hatched	# live
—	—	—						
Control Instant Ocean	A1	4	0	4	1	4	4	4
	B1	4	0	4	0	4	4	4
	C1	4	0	4	2	4	4	4
	D1	4	0	3	1	3	3	3
Caffeine .05 mg/ml	A2	4	0	3	1	3	3	3
	B2	4	0	3	0	3	3	3
	C2	4	0	3	2	3	3	3
	D2	4	0	3	2	3	3	3
Caffeine .25 mg/ml	A3	4	0	4	3	4	4	4
	B3	4	0	2	1	2	2	2
	C3	4	0	3	1	3	3	3
	D3	4	0	4	1	4	4	4
Caffeine 1.0 mg/ml	A4	4	0	2	0	2	0	1
	B4	4	0	4	1	3	1	1
	C4	4	0	3	0	3	0	3
	D4	4	0	3	0	3	0	2

*no class Thursday so the last day was 96 hpf

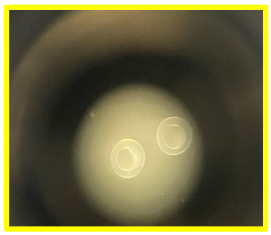


Figure 1

A1 (Instant Ocean): healthy, unhatched zebrafish (day 1)

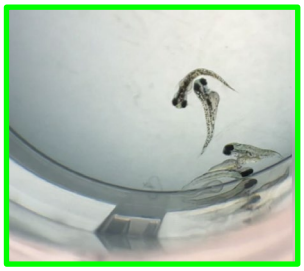


Figure 2

A3 (.25 mg/ml): excess somites (day 4)

Figure 3

B3 (.25 mg/ml): curved spine (day 4)

Figure 4

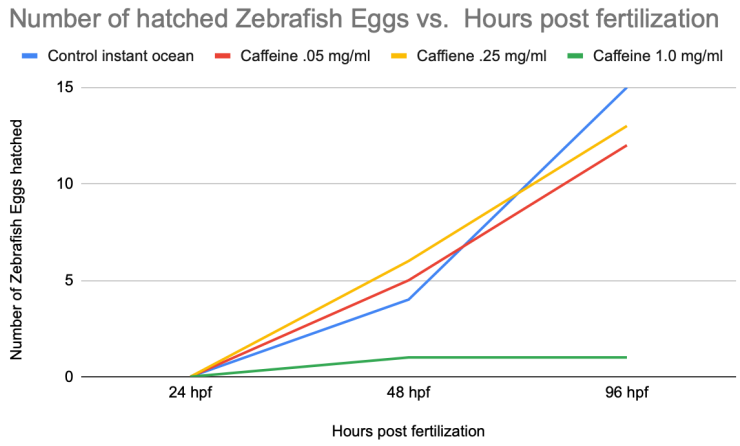
C2 (.05 mg/ml): curved spine, stunted growth (day 4)

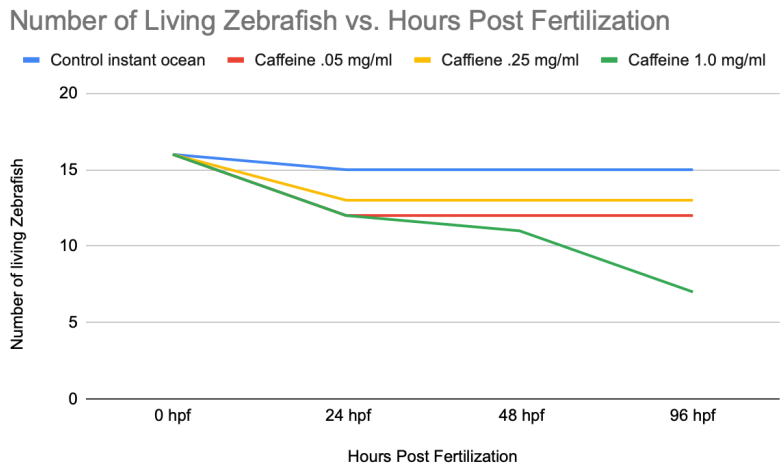
Figure 5

A1 (Instant Ocean): healthy, hatched zebrafish (day 4)

Day 4 physical development:

Control Instant Ocean	normal activity, no developmental issues
Caffeine .05 mg/ml	hyper, curved spines and stunted growth
Caffeine .25 mg/ml	hyper, curved spines and excess somites
Caffeine 1.0 mg/ml	extremely hyper





Statistical significance:

Chemical concentration	Mean	Standard deviation	T test (p-value)	Statistical significance
Control	3.75	0.50	—	—
.05 mg/ml	3.00	0.00	0.0240	Significant
.25 mg/ml	3.25	0.96	0.3903	Not significant
1.0 mg/ml	1.75	0.96	0.0100	Significant

The medium caffeine concentration group, .25 mg/ml, is not statistically significant since it's p-value is 0.3903, which is greater than .05. This means that, for the medium concentration group, the null hypothesis is accepted and a factor other than the caffeine concentration affected the zebrafish. On the contrary, the low and high concentration groups, .05 mg/ml and 1.0 mg/ml, were statistically significant since the p-values were 0.0240 and 0.0100, both of which are less than .05. This means that, for these two concentrations, the null hypothesis was rejected, and it was the caffeine which impacted the number of living and hatched zebrafish along with the physical development.

Results:

The caffeine content, whether it was high or low, affected the zebrafishes' growth and development significantly. Generally, the higher the caffeine content, the more likely the zebrafish egg would have some growth and development issues. For example, the group with the highest concentration of caffeine, group 4, had the most deaths and the lowest hatching rate. Since only 1 egg hatched in group 4, there was not a lot we could determine about developmental issues, but this fish was extremely hyper as compared to the fish in the lower concentration groups. From day 1 through day 4, the control group showed no signs of poor health, hyper behavior or developmental issues (see figures 1 and 5: ideal growth). The zebrafish's activity level increased with increasing caffeine concentration as noted in the "Day 4 physical development" chart. The eggs that did hatch, but had development issues, were from various concentration groups; no singular caffeine concentration presented itself as the most prone to causing these issues (see figures 2-4: examples of curved spines and excess somites). The "Number of Living and Hatched Zebrafish Embryos" data table shows a clear distinction between the control group and the 1.0 mg/ml caffeine concentration group. On the final day, 96 hours post fertilization, the control group had 8 more living zebrafish and 14 more hatched zebrafish as compared to the 1.0 mg/ml group. Although these two groups prove that as the caffeine concentration increases, the death rate increases, we cannot state this as true. This is because the .05 mg/ml group, which is the lowest caffeine concentration, had one more dead fish than the .25 mg/ml group. Our data also does not fully support the claim that as the caffeine concentration increases, the hatch rate decreases. It took a little more than 24 hours for the eggs to start hatching, but after that, the control group, the .05 mg/ml group, and the .25 mg/ml group all progressed with similar hatching rates. At the 96 hour mark, the control group had the least deaths and the most eggs hatched with a total of one death and 15 hatched after 96 hours. The .05 mg/ml group had 4 deaths and 12 hatched, but the .25 mg/ml group had 3 deaths and 13 hatched, which challenges our claim. The 1.0 mg/ml group, though, does support our claim as it had the most negative effects on the eggs with a total of 9 deaths and 1 hatched after 96 hours.

Discussion:

Through observing the results of the lab, we have concluded that the majority of our data proves our alternative hypothesis to be true, which states that an increase in the caffeine concentration will slow the process of zebrafish development and increase the number of zebrafish deaths. This hypothesis relates to our research because if caffeine concentrations have effects on zebrafish eggs, then caffeine consumption can have an effect on human embryos. Our

research indicates that an excess of caffeine can slow the development of embryos as well, so this experiment supports our claim and research to bolster it.

All of our groups, except for the .25 mg/ml group, reject our null hypothesis and therefore support our alternate hypothesis. This is supported by our P values. The .05 mg/ml p-value was 0.0240, which is less than .05 so it is statistically significant, meaning the null hypothesis is rejected and the caffeine concentration did impact the development, survival and hatching rates of the zebrafish eggs. The .25 mg/ml group's p-value was 0.3903 which is greater than .05. This means it is not statistically significant and the null hypothesis was accepted, meaning the caffeine did not factor into the results. Lastly, the 1.0 mg/ml group had a p-value of 0.0100, so similarly to the .05 mg/ml group, it is significant and rejects the null hypothesis. For the statistically significant groups, the higher the caffeine concentration, the lower the survival rate was (P value) and the slower the eggs hatched.

Our data does support our research and claim that caffeine stimulants the central nervous system and boosts one's energy levels. This is supported in the "Day 4 physical development" chart which indicates a direct correlation between increased amounts of caffeine and increased movement. Similarly, the .05 mg/ml and .25 mg/ml groups compared to the control group support the idea that caffeine can lead to birth defects and other developmental issues. In the case of this lab, that presented itself mainly through stunted growth and curved spines, but in the case of a human, this may be an issue such as nerve or heart disorders, issues sleeping or digesting food. Since the majority of our data accepts our alternative hypothesis, and all three of the groups with caffeine had more deaths and fewer eggs hatched than the control group, our data serves as a warning to children and mothers especially to be cautious when it comes to caffeine intake. It can hinder growth and development, lead to many negative side effects, and it has a greater effect the younger someone is, possibly delaying a birth, leading to a premature birth, or causing a miscarriage.

Our experiment could have abnormal data because we took out a zebrafish egg thinking it was dead, but later speculated that it still could have been living. This would explain the abnormalities in the statistical significance of the 0.25 mg/ml concentration. Another possible source of error is the fact that we used the same pipette for each of the solutions. Although it would have a very minimal effect, it still could have affected the concentration amount if there was any excess concentration left in the pipette. In addition, the small sample sizes might also have increased the margin of error and reduced the effect of the study through impacting the p-values and complicating the data analysis. To put things into perspective, one 8 ounce cup of brewed coffee typically contains 95 mg of caffeine, which is equivalent to .40 mg/ml, but in an 8 ounce cup of brewed Starbucks coffee, for example, there is 180 mg, meaning there is .76 mg/ml (Bjarnadottir, Adda). One normal brewed coffee has a slightly larger caffeine concentration as our third group, the .25 mg/ml. When companies make coffee and add extra caffeine, the concentration increases, and in the case of a cup from Starbucks, the concentration is slightly smaller than our fourth group, 1.0 mg/ml. Caffeine intake adds up quickly, so it is important that people watch their intake to avoid health risks.

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