The Effect of Caffeine on the Development of Zebrafish

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Abstract

It is known that when consumed, caffeine affects many living organisms in different ways. However, the specific effects that the amount of caffeine has on an embryo's development have always been uncertain. As a group, we observed the effects that increased concentrations of caffeine had on the development of zebrafish over the course of 3 days. In this experiment, we placed zebrafish eggs into wells with varying concentrations of caffeine, allowing us to qualitatively observe the development of zebrafish and quantitatively measure the number of eggs that hatched, remained unhatched, or died, and how the concentration of caffeine affected their survival. 8 zebrafish eggs were placed in wells consisting of 4 different categories: the control group (0.0 mg caffeine solution), a .05 mg/ml caffeine solution, a .25 mg/ml caffeine solution, and a 1.0 mg/ml solution. The average number of fish alive in the 1.0 mg/ml concentration was significantly less than those in the control group. At 72 hours post-fertilization, 1.0 mg/ml concentration averaged at .33 living fish per well, and compared to the average number of fish alive in the control group, which was 7.67 fish per well, this data is extremely significant. The increase in fish deaths as caffeine consumption increased signifies the detrimental effects caffeine has on embryos including human embryos. In addition, at 72 hours post-fertilization many fish in the 0.25 mg/ml of caffeine solution had visible deformities, such as curved spines, enlarged yolk sacks, and smaller size. These experimental results correlate with previous research in that higher consumption of caffeine leads to increased miscarriages for pregnant women, as well as an impact on epigenetic processes that may prevent embryos from growing to their full potential.

Purpose: To determine the effect of caffeine on the development of zebrafish.

Background research

Caffeine, despite being the most commonly consumed drug in the world, is proven to have adverse effects when consumed at high doses (Meredith, Steven, et al). It is a psychoactive drug that acts as an antagonist at adenosine receptors. Caffeine works by blocking endogenous adenosine, a naturally produced chemical responsible for slowing nervous activity and causing feelings of tiredness, from binding to its receptors, thus effectively preventing the effects of adenosine and acting as a stimulus. This potential to alter the chemical composition and function of the nervous system is the source of caffeine's impacts on the body (How Things Work: Caffeine).

While caffeine can be consumed through a variety of food and drink, the majority of caffeine consumption is attributed to beverages. Chocolate and other foods that contain cocoa contain caffeine, but they contribute negligible amounts to the diet. Caffeine is most commonly consumed through beverages, 96% of which are coffee, tea, and soft drinks. Out of these, coffee is by far the most popular source of caffeine, contributing substantially to overall intake. Typically, coffee contains more caffeine than other beverages, and it is the primary source of caffeine for adults. For children, carbonated soft drinks are the primary source of caffeine (Mitchell, Diane, et al)

Today, more than 90% of adults in the U.S. regularly consume caffeine, averaging around 200 mg per day. Many who regularly consume caffeine develop a dependence on the drug, and they are unable to cut down on caffeine use.

Excessive consumption of caffeine, while its adverse effects can affect anyone, is especially dangerous for pregnant women. 45% of women who are pregnant have developed caffeine dependence and are unsuccessful in their attempts to cut down caffeine use, and 77% of

women who are pregnant experience some form of caffeine withdrawal (Meredith, Steven, et al). Excess caffeine consumption during pregnancy is proven to be harmful to prenatal development, and in utero caffeine exposure has been proven to have long-term effects into adulthood and even transgenerational effects.

One of the most common chemicals that fetuses are exposed to is caffeine. Pregnant women consume, on average, 150 to 200 mg of caffeine per day. An estimated 60% of women, who often don't know that they are pregnant, consume caffeine during the first month of pregnancy. Caffeine consumed by the mother crosses the placenta and reaches the fetus with ease, as the half-life of caffeine is much longer in the placenta and fetus (12-24 hours) than it is in adults (2-4 hours). This is due to the absence of the enzyme CYP1A2, coded for by the cytochrome P450 1A2 gene (*CYP1A2*), in the fetus, causing the metabolism of caffeine to slow significantly, thus amplifying its effects (Fang, Xiefan, et al).

In utero caffeine exposure has been linked to babies being born small for gestational age and an increased rate of miscarriages (Fang, Xiefan, et al). In fact, drinking at least 3 cups of coffee or tea per day is associated with a high risk of miscarriage, and caffeine consumption during early pregnancy is more strongly related to miscarriages than alcohol or cigarette use (Dlugosz, L, et al). Studies in mice have also shown that exposure to caffeine in utero can result in changes in brain development and behavior, high blood pressure, and reduced cardiac function (Fang, Xiefan, et al).

In this experiment, zebrafish will be used to study the impact of caffeine on development. Many of the adverse effects of caffeine on the development of the human embryo, such as the increased risk for miscarriages and being born small for gestational age (Fang, Xiefan, et al), can be observed in zebrafish embryos. Zebrafish are ideal for studying human health because they

have many comparable features. In fact, humans as zebrafish are both vertebrae so they have many of the same major tissues and organs, and humans and zebrafish even share up to 70% of their genes (Why Use the Zebrafish in Research).

Hypothesis:

Alternative: An increase in caffeine concentration will cause adverse effects in the development of zebrafish.

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

Materials:

- 3x4 well plate
- Beaker with Instant ocean solution
- Beaker with 0.05 mg/ml caffeine solution
- Beaker with 0.25 mg/ml caffeine solution
- Beaker with 1.0 mg/ml caffeine solution
- 3 ml transfer pipette
- Methylene blue solution
- Large unmarked pipette for collecting zebrafish
- 8 zebrafish in each well (96 total)
- Dissecting microscope

Method:

Day 1:

1. 8 zebrafish eggs were added to each well of a 12 well plate
2. 2ml of instant ocean solution was added to the 3 wells in the first column (column 1; see fig.1 for reference). These are the control wells
3. 2ml of 0.05 mg/ml caffeine solution was added to the 3 wells in the second column (column 2; see fig.1 for reference). These are the low concentration wells
4. 2ml of 0.25 mg/ml caffeine solution was added to the 3 wells in the third column (column 3; see fig.1 for reference). These are the medium concentration wells
5. 2ml of 1.0 mg/ml caffeine solution was added to the 3 wells in the fourth column (column 4; see fig.1 for reference). These are the high concentration wells
6. Pipette was dipped in methylene blue solution and then dipped into each of the wells from column 1 to column 4, starting with the control group and going from low concentration to high concentration, ending with the high concentration wells (1.0 mg/ml of caffeine). Pipette was dipped back into the methylene blue solution only if necessary.
7. Well plates were put into incubator at 28.5 degrees Celsius overnight

Days 2-3:

1. Dissecting microscope was used to observe the number of fish alive and the number of fish hatched on all four days of observation. Quantitative data is recorded and placed into a data table.
2. Only on day 3, physical development was observed. Qualitative data is recorded.

3. Dead embryos were removed from the well plate using a pipette, starting from control group, then 0.05 mg/ml caffeine solution, then 0.25 mg/ml caffeine solution, then 1.0 mg/ml caffeine solution
4. Solutions were removed from each plate using a pipette (tilt the plate so the embryos settle to one side and remove the liquid from the top)
5. Steps 2-7 from day one were repeated

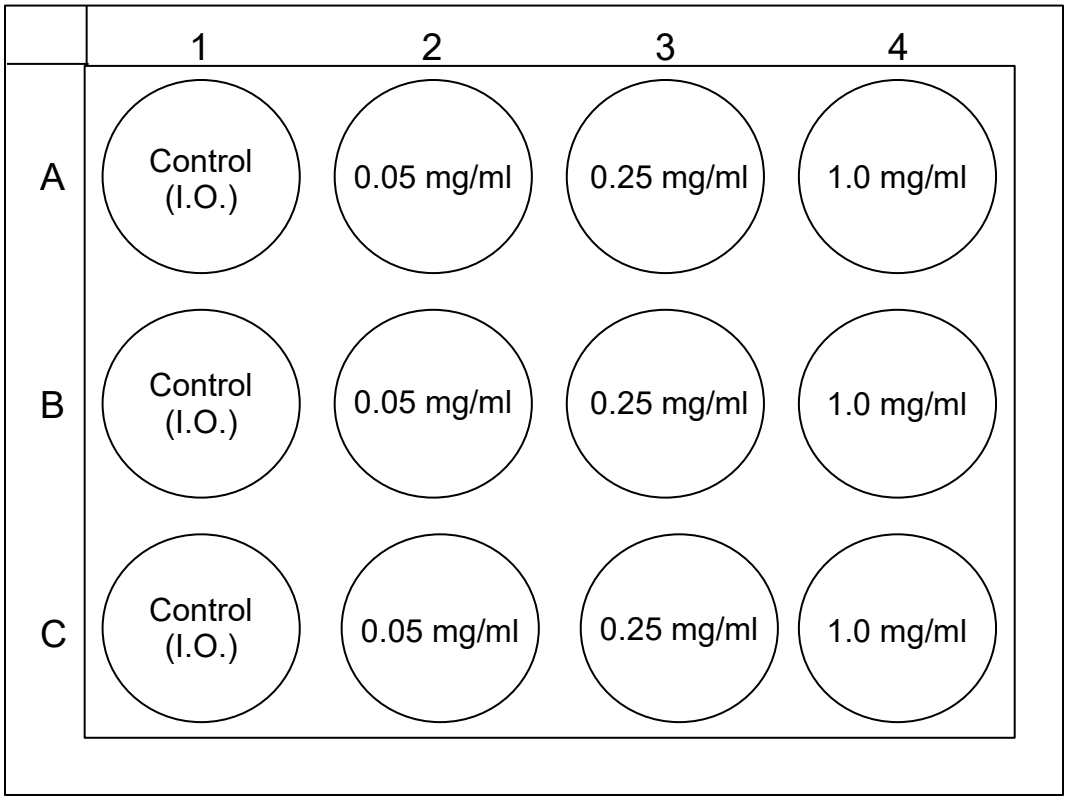

Fig. 1 well plate

Treatment	Well #	# of starting fish	48 hours post-fertilization		72 hours post-fertilization	
			# hatched	# live	# hatched	# live
Control Instant	A1	8	2	8	5	8
	B1	8	0	8	8	8

Ocean	C1	8	0	7	7	7
Caffeine .05 mg/ml	A2	8	0	8	4	8
	B2	8	0	8	8	8
	C2	8	0	8	8	8
Caffeine .25 mg/m	A3	8	4	8	8	8
	B3	8	4	8	8	8
	C3	8	5	8	8	8
Caffeine 1.0 mg/ml	A4	8	0	6	0	1
	B4	8	2	4	2	0
	C4	8	1	3	1	0

Fig. 2 Raw Data

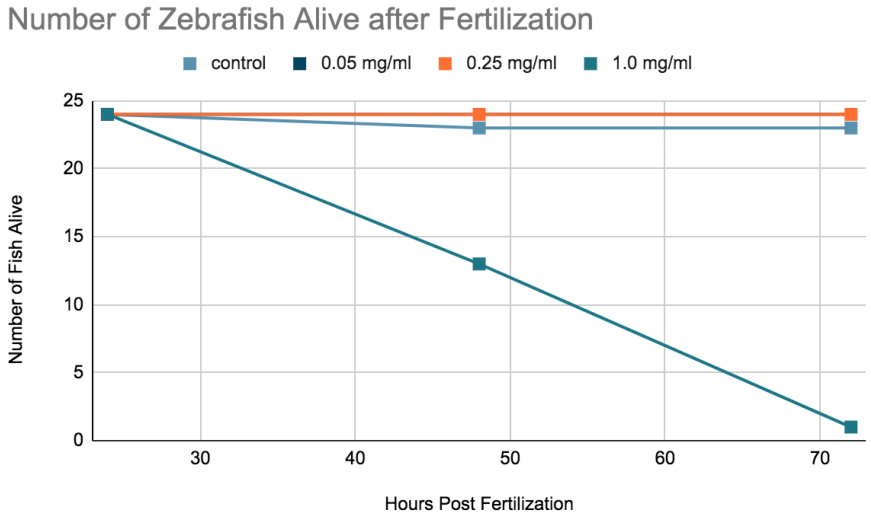

Fig. 3 Number of Zebrafish Alive vs. Hours Post Fertilization

The graph above illustrates the number of zebrafish alive at 24 hours, 48 hours, and 72 hours post-fertilization. Zebrafish in the 1.0 mg/ml solution experienced a steady decline in the number of fish alive, however, the data for the number of fish alive in 0.05 mg/ml and 0.25 mg/ml compared to the control group is insignificant.

*data for 0.05 mg/ml and 0.25 mg/ml is represented by the same points

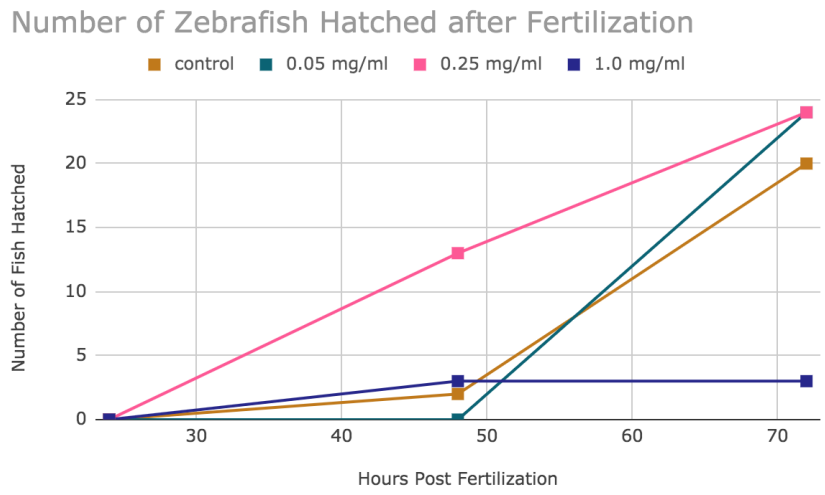

Fig. 4 Number of Zebrafish Hatched vs. Hours Post Fertilization

The graph above illustrates the number of zebrafish hatched at 24 hours, 48 hours, and 72 hours post-fertilization. The number of zebrafish in the 1.0 mg/ml solution that hatched was significantly small, and the number of zebrafish hatched in the 0.05 mg/ml, 0.25 mg/ml, and the control group increased steadily throughout the experiment.

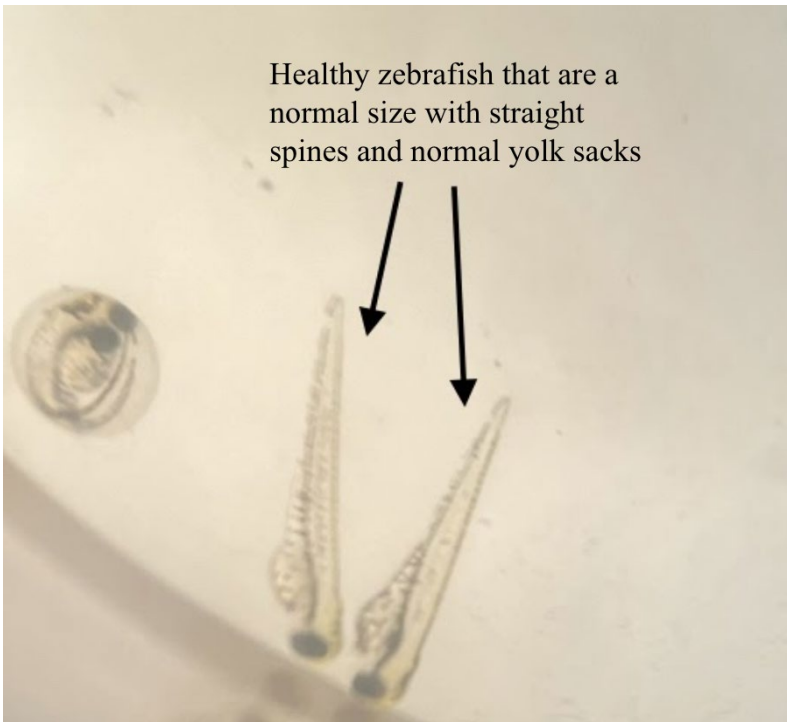

Fig. 5 Healthy zebrafish in well B1 (instant ocean solution)

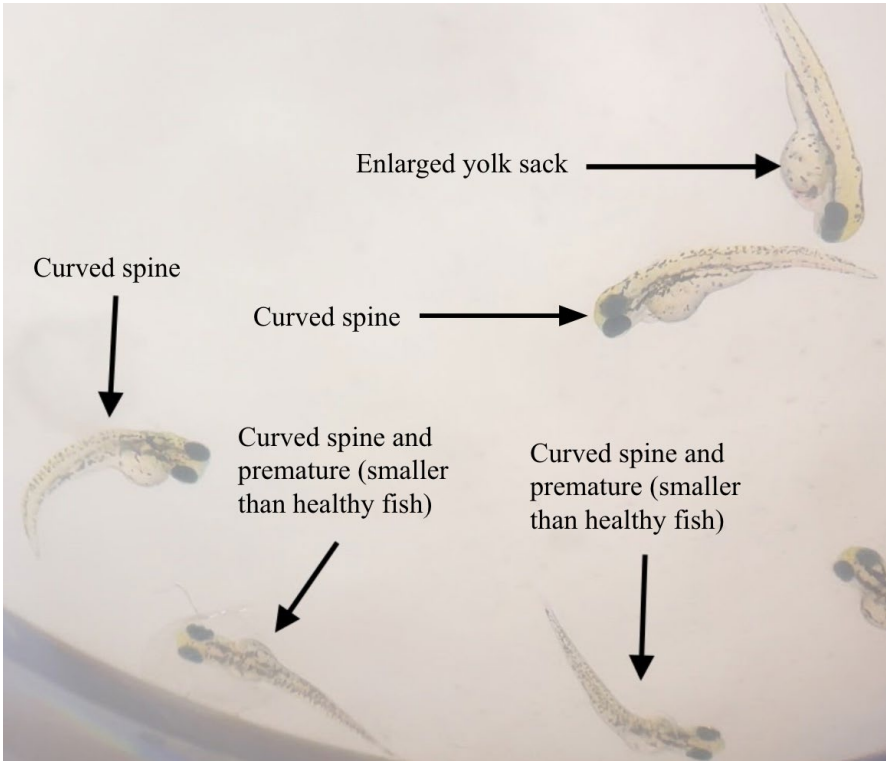

Fig. 6 Zebrafish with visible deformities in well A3 (0.25 mg/ml caffeine concentration)

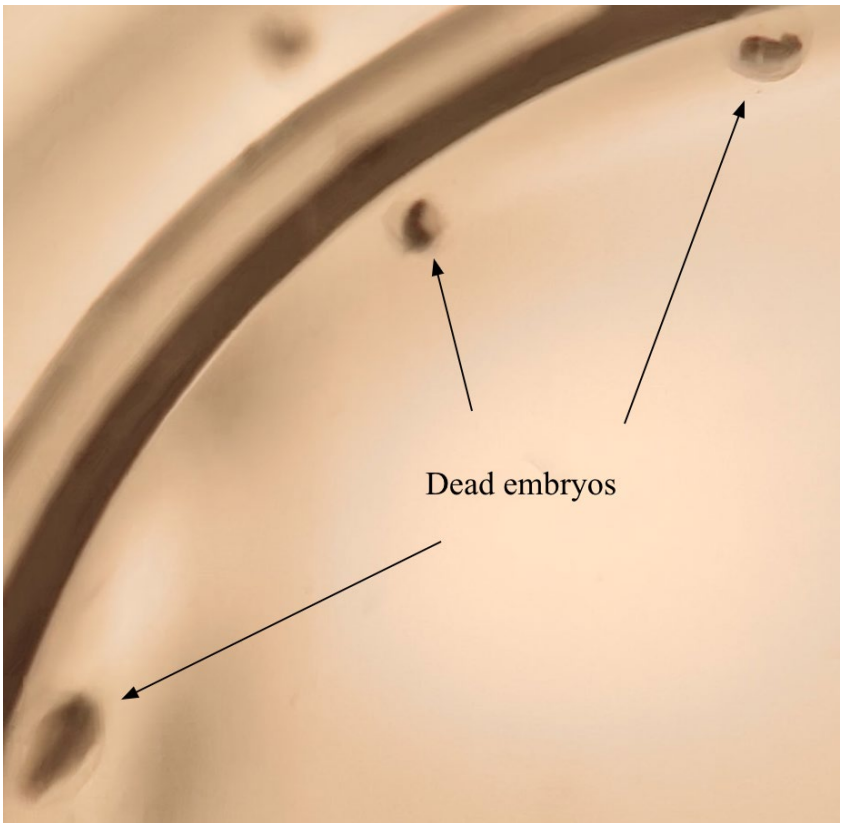

Fig. 7 Dead zebrafish in well B4 (1.0 mg/ml caffeine concentration)

Treatment concentration	Mean number of fish alive	Standard Deviation	P-Value (t-test)
control	7.67	0.58	N/A
0.05 mg/ml	8.00	0.00	0.3739
0.25 mg/ml	8.00	0.00	0.3739
1.0 mg/ml	0.33	0.58	<0.0001

Fig. 8 Statistical significance

The data for the low and medium concentrations of caffeine in comparison to the control group is not statistically significant, as they both have a p-value of 0.3739, which is much higher than

alpha (0.05). So, the null hypothesis, which states that an increase in caffeine concentration will have no effect on the development of zebrafish, is accepted for the low and medium concentrations. However, the data for the high concentration of caffeine compared to the control group is extremely statistically significant as the p-value, <0.0001, is much lower than alpha (0.05), so the null hypothesis is rejected. It can be concluded that for the high concentration, the hypothesis, which states that an increase in caffeine concentration will cause adverse effects in the development of zebrafish, is accepted.

Results

After administering the caffeine to the fertilized eggs, the effects throughout the following days showed that as the dosage of caffeine increased, the more significant the adverse effects on zebrafish development. This trend manifested in the eggs' death rates, hatching rates, and physical development.

In the control group (instant ocean solution), all but one fish survived the 72 hours after fertilization, and only five were left unhatched. For comparison, the death rate of the zebrafish in 0.05 mg/ml of caffeine and 0.25 mg/ml were rather insignificant, with both groups having all 24 zebrafish living 72 hours post fertilization. However, in the group with the highest concentration of caffeine, (1.0 mg/ml) only one fish remained by the last day, and most of the dead fish were still unhatched embryos (see fig. 7). To summarize said trend, the death rate increased with the concentration of caffeine (see fig. 3).

As for the physical development, there were significant deformities observed in the fish exposed to higher concentrations of caffeine, especially 0.05 mg/ml and 0.25 mg/ml caffeine solution. Similar to the trend in death rate, the higher the concentration of caffeine, the more

significant the negative impacts on physical development. Figure 6, which shows the 0.25 mg/ml well, the zebrafish's spines are visibly curved, and noticeably smaller than the healthy fish seen in the control group (see fig. 5). In the well with 0.05 mg/ml, similar effects were observed; however, they were not as significant. These physical abnormalities can only be observed in living fish, hence why the dead fish in the highest concentration (1.0 mg/ml) did not exemplify any physical deformations.

Compared to the control group, at low concentrations of caffeine (0.05 mg/ml) hatching rate of the zebrafish embryos was virtually unaffected, at medium concentration (0.25 mg/ml), hatching rate increased, and at high concentration (1.0 mg/ml) hatching rate decreased due to the high death rate. As illustrated by figure 4, at 72 hours post-fertilization 20 out of 24 total eggs in the instant ocean solution had hatched, 20/24 eggs from the 0.05 mg/ml caffeine solution hatched, all the eggs in the 0.25 mg/ml solution hatched, and only 3/24 eggs from the 1.0 mg/ml solution hatched. In addition, the eggs in the 0.25 mg/ml solution hatched at a quicker speed than those in the control group; at 48 hours post-fertilization only 2 of the eggs in the control group had hatched, but 13 eggs from the 0.25 mg/ml solution had hatched.

Discussion

An increased concentration of caffeine caused adverse effects on the development of zebrafish. First, high concentrations of caffeine caused an increased death rate in zebrafish, as shown by the graph in figure 3. 23 out of the 24 zebrafish placed in a solution of 1.0 mg/ml of caffeine had died 72 hours post-fertilization. The majority of these fish died before they even hatched. Figure 7 illustrates dead zebrafish embryos that did not hatch.

In addition, caffeine exposure caused many qualitatively observed deformities during the development of zebrafish. By 72 hours post-fertilization, only 1 out of 24 zebrafish in the instant

ocean solution had died, and the rest were alive and healthy with no visible deformities, as shown in figure 5. On the other hand, the zebrafish that were placed in wells with a 0.25 mg/ml caffeine concentration were smaller in size and had many visible deformities such as curved spines and enlarged yolk sacs, as shown in figure 6. With the .25mg/ml all the eggs hatched and were alive by 72 hours but were not healthy like the Instant Ocean Solution zebrafish.

In the wells that had the concentration of 1.0 mg/ml all of the fish died which is similar to how in the human body when someone consumes high doses of caffeine during pregnancy it can lead and is linked to miscarriages. In addition, the zebrafish that were in the concentration of .25mg/ml of caffeine hatched very early compared to those in the control group, as shown by figure 4, and they were a lot smaller compared to the healthy zebrafish that were in the Instant Ocean Solution, as shown in figure 6. This part of the experiment is similar to how in utero caffeine exposure is linked to babies being born small for their gestational age.

One source of error that occurred during this experiment was that when removing dead fish from well B4 at 48 hours post-fertilization, two fish were lost in the pipette. While it was known that one of the fish was dead, the other fish was still alive. Losing the living zebrafish prevented it from accurately being observed, and it potentially caused an artificially high death rate. A limitation from this lab is that zebrafish eggs that were never fertilized could have been placed in the wells. This would have caused an artificially high death rate. In addition, this error could have diminished the significance of the results by causing zebrafish embryos to die as a result of never being fertilized, rather than dying as a result of being exposed to caffeine.

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