Effect of 5-Hour Energy on Zebrafish Embryos

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ABSTRACT: The development and survival of zebrafish embryos raised in the presence of varying concentrations of the energy drink, 5-Hour Energy, were examined. After fertilization occured, 20 eggs were each exposed to 1 mg/ml of 5-Hour Energy caffeine, 0.25 mg/ml of caffeine and 0.05 mg/ml. Twenty-four hours post-treatment and approximately 28 hours post-fertilization resulted in 100% fatality for the concentrations of 1 mg/ml of caffeine and 0.25 mg/ml. However, all embryos treated with 0.05 mg/ml caffeine survived. Over the following 72 hours these zebrafish slowly died, leaving only 55% surviving to the final day. Since zebrafish embryos can be compared with human embryos, these results show that 5-Hour Energy is harmful, especially in high concentrations. These results suggest that pregnant mothers should avoid consumption to protect the embryo.

Background

Understanding how different substances affect the growth of a living organism is something that has been highly researched for decades. More specifically, caffeine is commonly labeled as a substance harmful to the development of an embryo. Research shows that during human pregnancy, caffeine can cross the placenta membrane and impact the developing embryo (Momoi, Tinney, Liu, et al., 2007). The absorption of caffeine puts the embryo at a higher risk of death due to the exposure. (Momoi, Tinney, Liu, et al., 2007). These risk factors have lead to a recommended limit to how much caffeine should be consumed by the average person, 400mg/day (United States Food and Drug Administration, 2018). In addition to this recommendation, although caffeine intake is not advised, studies have shown that women should be consuming less than 300 mg/d per day, with any more possibly leading to obstructions in development of the fetus, and caffeine withdrawals after birth in extreme cases (Morgan, Koren, Bozzo, 2013). This lab further investigates the most severe consequences of not following these restrictions and consuming excess caffeine in the form of 5-Hour Energy during a pregnancy, and its effects on the death rate of an embryo.

By exposing zebrafish embryos to caffeine, an environment is created that simulates the effects of caffeine of a developing human in utero. Conclusions determined by zebrafish embryonic research can be applied to effects on human embryos. This is true because of their similar structures; they have many of the same major organ systems and structures, including spinal cord, and 70% of the same genes (Burke, 2016). By studying the effects on zebrafish, the results can be applied to humans because of the physical similarities. Additionally, zebrafish are highly available and they respond clearly in ecotoxicology studies (Scholz, Fischer, Gündel, et al., 2008). This allows a magnitude of experiments to be done with clear results, allowing accurate comparisons between humans and zebrafish to be drawn. Lastly, zebrafish typically yield accurate results when the surrounding environment of the embryo is changed; in this lab's case that is the surrounding caffeine concentration. This allows the conclusions of this lab's data,

about the effects of 5-Hour Energy on zebrafish embryos, to draw parallels to the effects of 5-Hour Energy on human embryos.

5-Hour Energy is a popular choice for those who want a caffeine rush in the middle of the day. One serving, or one bottle, contains exactly 200mg of caffeine (Hall, 2012). In comparison, an 8 oz cup of coffee has about 135 mg of caffeine. 5-Hour Energy also contains additives along with the high caffeine content (Morgan, Koren, Bozzo, 2013). Some of the most crucial include high concentrations of taurine, niacin, vitamins B6 and B12, and folic acid in addition to the caffeine (Morgan, Koren, Bozzo, 2013). Although there was little data of its effects on zebrafish embryos, there was a study performed on the exposure to taurine on fertilized chicken embryos. Research suggested, that after a 15 day incubation period there was an increased concentration of taurine in the brain and heart (Van Gelder, Bélanger, 1988). Additionally they found that when ready to hatch, some chicks were unable to break out of their shell and some had a delayed hatching process (Van Gelder, Bélanger, 1988). If taurine effects the zebrafish embryos a similar way, it could possibly also lead to an increased death rate of the embryos because of a lack of ability to hatch.

These chemicals lead to the research questions: how does the amount of 5-Hour Energy exposed to zebrafish affect their survival rate, and how do additives play a role in the increasing or decreasing that value? By comparing 5-Hour Energy to pure caffeine at the same caffeine concentrations, it allows for the analysis of how 5-Hour Energy affects the death rate of zebrafish embryos. The negative control is a standard stock solution that represents how healthy embryos develop. It is used a base for this comparison with the experimental group. In the lab, zebrafish embryos were exposed to either a pure caffeine solution or a 5-Hour Energy solution with the concentration of caffeine being 1 mg/ml, 0.25 mg/ml, or 0.05 mg/ml. They were exposed over a 96 hour period, and the number of deaths was recorded every 24 hours after the initial treatment. The hypothesis is, if the concentration of 5-Hour Energy increased, then the death rate would also increase, because it would lead to a higher exposure and higher uptake of caffeine which a substance that is harmful during stages of embryonic development. Although the data collected was not statistically significant, the hypothesis held true when compared to those exposed to pure caffeine. All fish exposed at the two highest concentrations of 5-Hour Energy died after the first 24 hours of treatment, and the majority of 5-Hour Energy fish exposed at the lowest concentrations died in the 96 hours that data was collected. Pure caffeine fish experienced higher survival rates across the board. This ultimately lead to the conclusion that 5-Hour Energy is not only dangerous while an embryo is developing, it is more deadly than pure caffeine.

Materials and Methods

Materials

In order to properly execute this lab, specific materials are needed. To create the proper solutions to expose to the embryos, Instant Ocean, one bottle of 5-Hour Energy (any variant with 200mg of caffeine), and a pure caffeine solution are needed. Glass bottles that can be sealed, labeled and stored in a refrigerator are recommended for the storage of the solutions. Additional materials needed include, freshly fertilized zebrafish embryos to be exposed to the solutions, access to an incubator for the storage of the embryos, and a large amount of disposable pipettes with narrow and wide openings to remove dead embryos and replace solutions. Lastly, a 12 well falcon dish and a dissecting microscope are needed.

Chemicals

In this experiment, only two potentially unsafe chemicals were used: caffeine found present in 5-Hour Energy and in pure caffeine. 5-Hour Energy is approved by the Food and Drug Administration, which is a government department that sets quality standards and regulations for food and drug items sold the United States (5-Hour Energy, 2017). Thus, 5-Hour Energy, although not consumed in this lab, is considered safe to ingest and not directly harmful to human health at its given amount. For this reason, the majority of chemical safety standards while handling this substance need not be observed during lab. Similarly, the highest concentration of pure caffeine solution that was used was 1 mg/ml which is far lower than the recommended maximum consumption of caffeine per day of 400 mg (United States Food and Drug Administration, 2018). Once again, the concentration used is far lower than that any amount considered harmful to humans, and why the majority of chemical safety standards were not followed for pure caffeine as well.

The various concentrations that were tested on the embryos were created by serial dilution in this lab. In a single bottle of 5-Hour Energy, there is 200 mg of caffeine (Hall, 2012). One entire bottle of 5-Hour Energy (57 ml) was combined with 143 ml of Instant Ocean in order to create a 1 mg/ml stock solution of caffeine. From this original solution, additional Instant Ocean was added to attain concentrations of 0.25 mg/ml, 0.20 mg/ml, 0.10 mg/ml and 0.05 mg/ml. A similar process was followed with the pure caffeine solution. A base of 1 mg/ml was provided at the start of the experiment. Instant Ocean was also added to this base in order to attain concentrations of 0.25 mg/ml and 0.05 mg/ml. All solutions were stored a refrigerator when not in use to prevent solution separation or chemical decomposition, and all were shaken before use each day.

Set Up

The following procedure is based off of research for the University of Wisconsin-Milwaukee (2018), in a lab entitled “Zebrafish As Models: Studying the Effects of Environmental Agents on

Human Health." The initial premise of the UW-Milwaukee lab is to display the effects of foreign substances on the embryonic development of zebrafish in an attempt show how the substances may affect human development. The purpose of that lab was modified, changing the experimental substance to 5-Hour Energy. The lab was designed to eliminate as many inconsistencies as possible. The first step was dividing the falcon dish. The dish itself had 12 compartments total with 3 rows and 4 columns. The first 2 columns became control dishes; the first was a negative control with exclusively the Instant Ocean solution. The second was a pure caffeine solution with the concentrations of 1 mg/ml, 0.25 mg/ml, and 0.05 mg/ml, respectively going down the column. The next two columns were the experimental groups. They both initially used the same concentrations as the control caffeine row except with 5-Hour Energy solution, instead of caffeine as the solute. (The concentrations of the top two wells in those columns eventually change to 0.20 mg/ml, 0.10 mg/ml, respectively, as the lab continued due to a desire to do a pilot test with new concentrations after the 1 mg/ml and 0.25 mg/ml concentrations died. For further explanation of this process, please reference the "Pilot Lab" and in the "Method and Materials" and "Discussion" sections of this lab)

Once the organization of the falcon dish was determined, ten fertilized embryos were placed in each well. These embryos were approximately four hours old, after fertilization occurred earlier that morning. In order to make sure they were all alive and fertilized it was necessary to look under a microscope and see the sac on the outside of the egg. If that sac was not there or the inside of the egg was cloudy, it was necessary to remove and replace those eggs, for they were either not fertilized or dead.

After the ten living and fertilized eggs were placed in each well, the stock solution had to be removed using a small pipette and replaced halfway full with the predetermined solutions using a clean pipette. This process, while tedious, ensured that there was no cross contamination between wells. Finally, after the set up was completed, the eggs went into the incubator so they could develop, and the solutions went into the refrigerator to prevent from decomposition.

Procedure During Lab

Every 24 hours following lab set up, a daily procedure was followed to ensure that the embryos were in a clean environment. The number of dead and alive embryos per concentration were recorded on a spreadsheet and a picture of the experimental wells were taken. This was accomplished by using a dissecting microscope to analyze each well. Dead embryos were identified by clouding and loss of transparency. As embryos hatched, death was determined by black pigmentation. All dead were removed using a large pipette after being recorded. Once completed, the old solution was removed using a thin pipette, a new one for each type of solution, and then those solutions refilled in their respective wells also using a new pipette for each solution to prevent cross contamination. Following this, the falcon dish was returned to the

incubator, and the solutions back to the refrigerator. This process continues until 96 hours post-fertilization was completed.

Data Analysis

After the number of dead are recorded after the 96 hour period, calculate the percent that survived for every 24 hour period for every different exposure of caffeine by taking the amount that survived each day over the starting number for that category. Then compare the percentages. Look for the lowest survival rate to determine which concentration was the most deleterious. Additionally, compare survival rates between identical concentrations 5-Hour Energy and pure caffeine; the lowest percentage was more deadly to the embryos. Ideally, the survival rates should be determined by well, meaning if there was two wells of 5-Hour energy at 0.05mg/ml, the survival rate in both wells would be analyzed independently. This would allow for multiple data points for each concentration, and thus a "p" value and the standard of the mean could be determined to see if the data is statistically significant.

Pilot Lab

While gathering data, there was an attempt to identify the concentration of 5-Hour Energy that would be considered dangerous, creating a threshold that could be considered safe. The highest concentrations of 1 mg/ml and 0.25 mg/ml were ruled out immediately because everything died in those wells after 24 hours. So, solutions of 0.20 mg/ml and 0.10 mg/ml were added to the experiment and tested as well along with the original .05 mg/ml. Ideally, freshly fertilized eggs would have been exposed to the new concentrations, but because eggs that had been fertilized 24 hours before were the only eggs available, that is what was used. Like previously mentioned, the goal was to find a threshold concentration that would provide a maximum amount of 5-Hour Energy that could be considered safe for consumption. Theoretically, the process of having freshly fertilized eggs and exposing them to various concentrations and ruling out the most harmful to find one acceptable concentration would have likely accomplished this. Though, because the pilot lab labs were started 48 past the initial fertilization period, this pilot lab was inconclusive.

Results

Experimental Design

In this experiment, groups of 20 fish were exposed to solutions of caffeine and 5-Hour energy. The concentrations tested for each were 1.0 mg/ml, 0.25 mg/ml and 0.05 mg/ml of caffeine which is the independent variable. Each day the total amount of alive fish were recorded per concentration of solution which is the dependant variable.

Control Group

For the simplicity of the experiment, the positive control solutions were referred to as "pure caffeine," as not to confuse it with the caffeine found in 5-Hour Energy. Confirming the results found by the aforementioned studies towards this lab was necessary as a basis of comparison. This was accomplished by exposing 10 embryos to 1 mg/ml, 0.25 mg/ml and 0.05 mg/ml of pure caffeine [Figure 1]. For 1 mg/ml, the results were exactly as expected. Over the first 48 hours, 50% of the embryos survived and by the termination of the lab after 96 hours, only 20% [Figure 2] had survived. The embryos exposed to 0.25 mg/ml caffeine showed different and unexpected results. Their sample maintained a 100% [Figure 3] survival rate through 48 hours. However, by 96 hours, 50% of the original population was still alive[Figure 2]. At a concentration of 0.05 mg/ml, the survival rate dropped to 80% [Figure 2] over the first 48 hours and remained unchanging though the termination of the experiment.

Also included was a negative control with 0 mg/ml caffeine. The sample size for this control was 30 fish divided up over 3 wells in the falcon dish [Figure 1]. Over the first 24 hours, 2 of the 30 embryos died lowering the survival rate to 93% [Figure 2]. No other fish perished over the experiment maintaining that 93% to termination.

Number of Surviving Fish in Caffeine over 96 Hours

Number of living fish Caffeine	**Day of Treatment**	**24 hours Post**	**48 Hours Post**	**72 Hours Post**	**96 Hours Post**
1 mg/ml	10	6	5	4	2
0.25 mg/ml	10	10	10	7	5
0.05 mg/ml	10	9	8	8	8
0 mg/ml	30	28	28	28	28

Figure 1 Raw data of how many fish survived per day in each concentration of caffeine. The original starting amount was 10 embryos for 1 mg/ml, 0.25 mg/ml and 0.05 mg/ml. The 0 mg/ml started with 30 total embryos.

Percent Survival of Fish in Caffeine

Final Survival Rate Caffeine	**96 Hours Post Treatment**
1 mg/ml	20%
0.25 mg/ml	50%
0.05 mg/ml	80%
0 mg/ml	~93%

Figure 2 Final percentage of zebrafish exposed to caffeine solution alive though the entire experiment. Calculated by dividing the number alive after 96 hours by the number of original then multiplying it by 100.

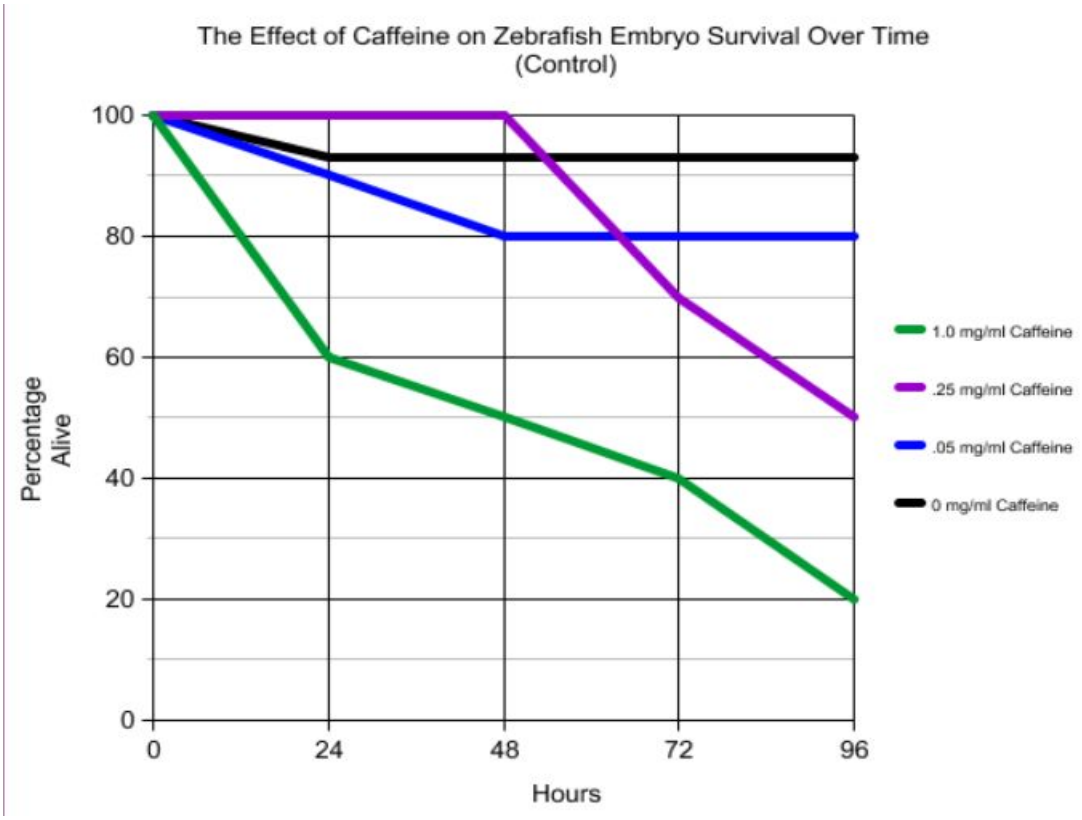

Figure 3 Graph depicts the percent living of zebrafish exposed to caffeine solution over time. Data was recorded every 24 hours after the first exposure.

5-Hour Energy Group

*****The .20 mg/ml and .10 mg/ml concentrations of 5-Hour Energy Caffeine were administered 24 hours post fertilization and must be discarded from this research.

Originally, the embryos were to be tested with 1 mg/ml, 0.25 mg/ml and 0.05 mg/ml concentrations of 5-Hour Energy caffeine. In total, 20 embryos were divided over 2 dishes for each concentration. However, 24 hours post treatment, all fish in both the 1 mg/ml and 0.25 mg/ml concentrations died [Figure 6]. The only group to survive the first 24 hours was the 0.05 mg/ml solution [Figure 6]. As the experiment progressed, the percent alive of this solution slowly dropped and on the final day it was 55% [Figure 5]. Additionally, there was a physical change in these fish. By the 96th hour post-treatment, 11 out of the original 20 were alive [Figure 4] yet none had hatched. They were clearly alive and semi-developed, but seemed to have stopped changing or drastically slowed somewhere around 48 hours in. These results were not observed in any other concentration of 5-Hour Energy caffeine or control caffeine yet was observed in both wells of the experimental.

Number of Surviving Fish in 5-Hour Energy over 96 Hours

	Day of Treatment	**24 Hours Post**	**48 Hours Post**	**72 Hours Post**	**96 Hours Post**
1 mg/ml	20	0	0	0	0
.25 mg/ml	20	0	0	0	0
.05 mg/ml	20	20	18	15	11

Figure 4 Raw data of how many fish survived per day of each concentration of 5-Hour Energy caffeine. The original starting amount was 10 embryos for 1 mg/ml, 0.25 mg/ml and 0.05 mg/ml. The 0 mg/ml started with 30 total embryos.

Percent Survival of Fish in 5-Hour Energy

Final Survival Rate 5-Hour Energy	**96 Hours Post Treatment**
1 mg/ml	0%
.25 mg/ml	0%
.05 mg/ml	55%

Figure 5 Final percentage of zebrafish exposed to 5-Hour Energy solution alive though the entire experiment. Calculated by dividing the number alive after 96 hours by the number of original then multiplying it by 100.

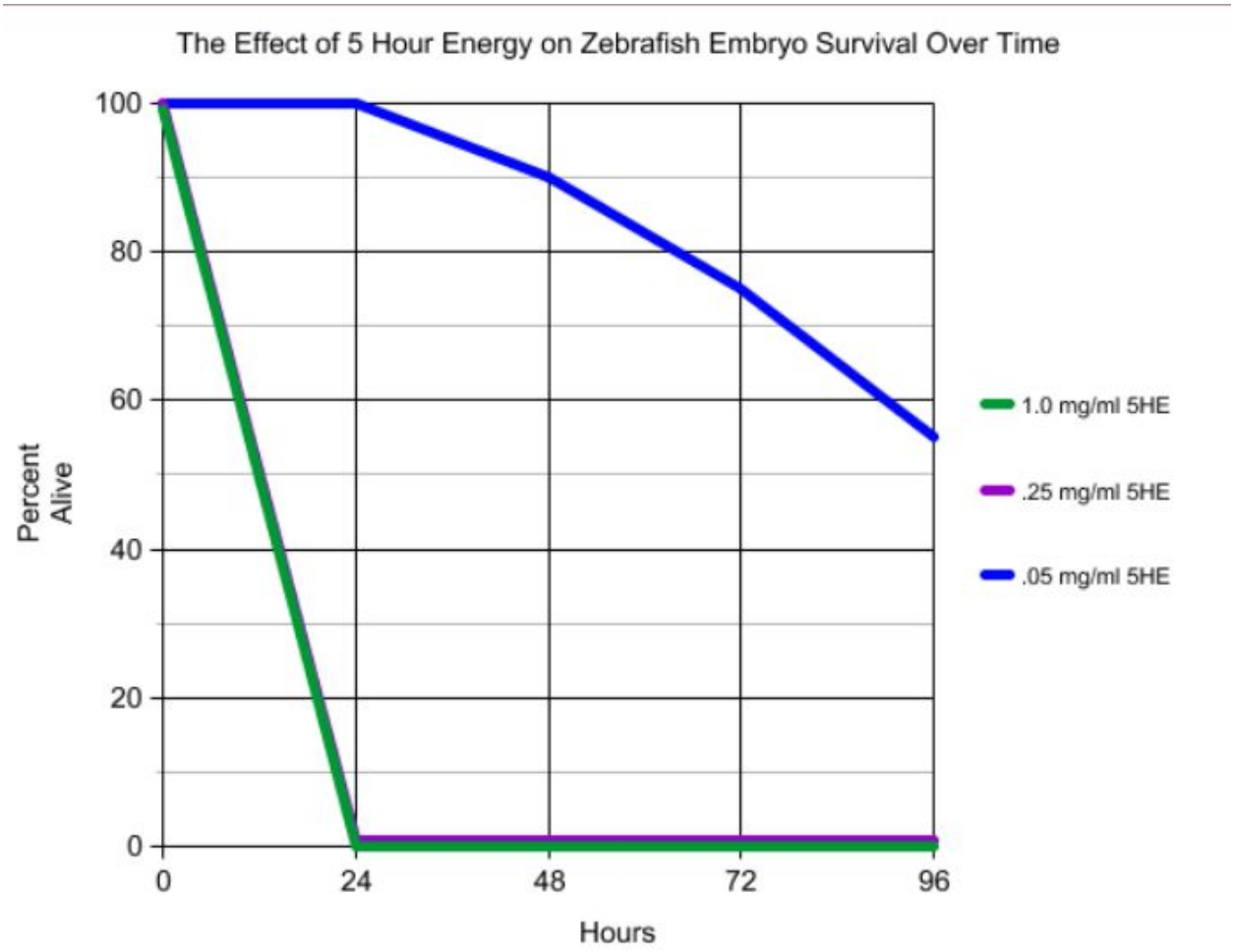

Figure 6 Graph depicts the percent living of zebrafish exposed to caffeine solution over time. Data was recorded every 24 hours after the first exposure.

Discussion

Death Rate

The results of this experiment have several possible extrapolations to humans. Firstly, and most obviously, is that caffeine in any form is harmful to humans. In both the positive controls and the experimental group, the caffeine content did lower survival rates. This is in accordance with the hypothesis because in both the control and experimental, the more caffeine exposure, the lower the survival rate. It is clear that if there is more caffeine present during those crucial development stages, the embryo is less likely to survive.

Instant Ocean

The negative control, embryos placed in the Instant Ocean solution, had two deaths within the first 24 hour period post-treatment. No other deaths were recorded for this group besides the initial two that died. In all likelihood, these preliminary deaths can be attributed to some other variable that was uncontrollable or perhaps they simply were weak or damaged in some way not visible to the eye. Finally, there is also the possibility that they were already dead but were not noticed on the first day when they were placed in the wells. This data was ultimately not used in the final comparison of the effects of caffeine and 5-Hour Energy. However, the final survival rate of 93% is drastically higher than any other group showing that no caffeine is ideal.

0.05 mg/ml 5-Hour Energy Well

An important comparison can be made between the 1 mg/ml and 0.25 mg/ml concentrations of the experimental and control groups. Although they had the same level of caffeine, the 5-Hour Energy solution caused total death for all embryos after only 24 hours. However, the control simply killed them gradually over several days. This does agree with the hypothesis, that higher levels of caffeine cause higher amounts of death, but why was there such a discrepancy between control and 5-Hour Energy? There are several explanations for why this may have occurred. Firstly, and most likely was that 5-Hour Energy is not a pure substance. Although the 1 mg/ml concentrations were identical caffeine wise, there were a whole host of other chemicals, including flavorings, dyes and preservatives present in the 5-Hour Energy (Morgan, Koren, Bozzo, 2013). Any one of those could have had a detrimental effect on the embryos. Most suspicious of these chemicals present in 5-Hour Energy is taurine, which has been linked to several issues involving embryo development as previously mentioned in the background. In chickens, it causes delayed hatching and issues breaking the shell (Van Gelder, Bélanger, 1988). If it can affect a chicken in this way, it could also affect a zebrafish and kill them or prevent them from hatching.

The aforementioned “stunted growth” potentially caused by taurine may have appeared in this experiment. The only embryos that were introduced to the 5-Hour Energy solution to survive beyond the 24 hour mark was the lowest concentration, 0.05 mg/ml. While it is true their

population decreased to 55% [Figure 5] of the original population, the more shocking results was the lack of development. By the final day, not a single of the remaining 11 had hatched [Figure 4]. They were clearly alive and most appeared fully developed, however they remained in their shells when all should have hatched between approximately 48 and 72 hours (Kimmel, Ballard, Kimmel et al. 1995). At 96 hours, all or most of the fish should have been completely developed, yet not one of the survivors did. Figures 7, 8, and 9 show the lack of development, with the eggs showing no physical changes between 72 hours, and 96 hours post-fertilization. In contrast, Figure 12 shows that the embryos should have hatched and matured into mobile and fully formed fish before 96 hours. This leads to the conclusion that, although death was not as prevalent as it was at the higher concentrations of 5-Hour Energy, the exposure of of zebrafish embryos on the day of fertilization to 5-Hour Energy stops the development process of the embryo and prevents it from hatching.

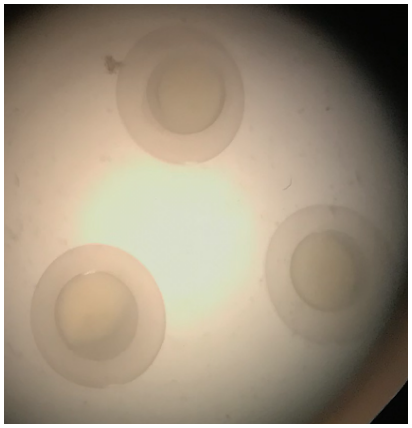

Figure 7 This image depicts zebrafish embryos in the 0.05 mg/ml concentration of 5-Hour Energy the day of fertilization

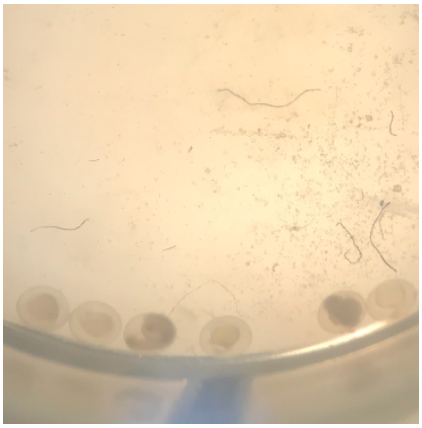

Figure 8 This image depicts zebrafish embryos in the 0.05 mg/ml concentration of 5-Hour Energy the 72 hours post fertilization

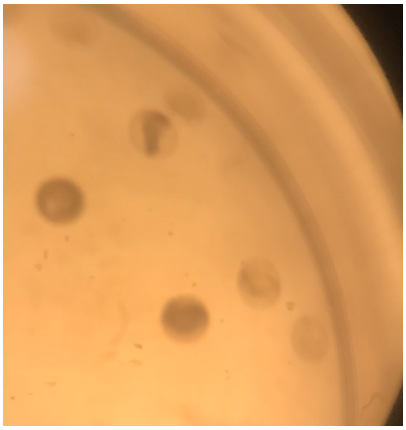

Figure 9 This image depicts zebrafish embryos in the 0.05 mg/ml concentration of 5-Hour Energy the 96 hours post fertilization

Pilot Lab

After the first 24 hours, two of the experimental groups perished completely: the 1 mg/ml and 0.25 mg/ml concentrations. The decision was made to find a specific tolerance region of 5-Hour Energy solution between 0.25 mg/ml and 0.05 mg/ml. The concentrations of 0.20 mg/ml and 0.10 mg/ml were decided upon. This data, though included in this paper, cannot be compared with the original set of data due to the extreme discrepancy between when the fish were exposed to the solutions. The first batch was exposed extremely early, mere hours after fertilization, while the second batch was over 24 hours post-fertilization. This gap is too great to fairly compare the results. However, it can be assessed individually and separately from the other experiment. Despite being immersed in relatively high concentrations, none of the embryos in 0.20 mg/ml

[Figure D] and 0.10 mg/ml [Figure E] solutions died and all were perfectly developed and hatched. They had the same appearance and developmental stage as the unexposed embryos in Instant Ocean solution [Figure F]. This brings up the idea that caffeine is most detrimental to zebrafish in the very early stages of development and if they are exposed later, the effects may be minimized. This very same occurrence is visible in humans as the majority of deformities, some fatal, occur in the weeks 0-8 after fertilization. For humans, this is considered the embryonic stage. As the embryo becomes a fetus in week 8, the incidence of deformities during pregnancy falls from 10% to 1% (Adé-Damilano, Celio, 2008). Since zebrafish are oftentimes analogous to humans, there is some evidence to say that caffeine is most damaging to a human embryo in its first 2 months of life.

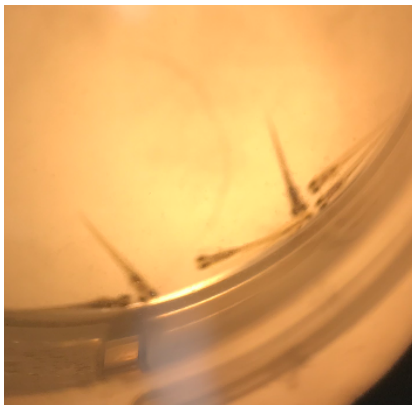

Figure 10 This image depicts zebrafish embryos in the 0.2 mg/ml concentration of 5-Hour Energy the 96 hours post fertilization

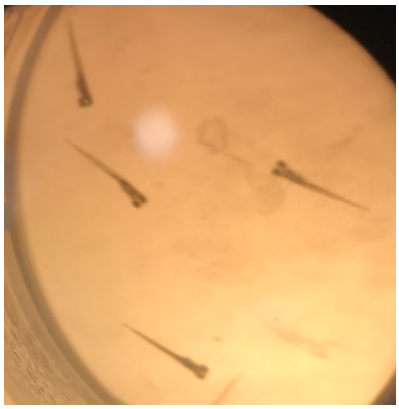

Figure 11 This image depicts zebrafish embryos in the 0.1 mg/ml concentration of 5-Hour Energy the 96 hours post fertilization

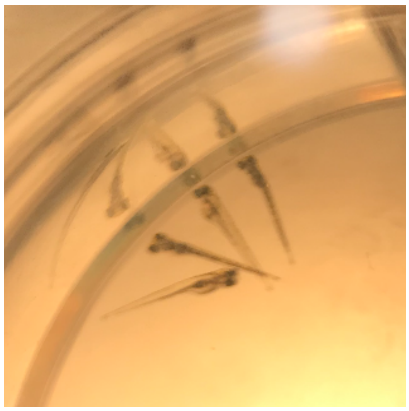

Figure 12 This image depicts zebrafish embryos in the Instant Ocean solution 96 hours post fertilization

Sources of Error

While not in use during lab, both the 5-Hour Energy and caffeine solutions were stored in a refrigerator in order prevent deterioration of the caffeine in them. However, this may not have been entirely effective, especially with the 5-Hour Energy. At 48 hours post treatment, an unknown white solid had formed in the 1 mg/ml container. When shaken, the solid appeared to dissolve back into solution, but by the following 72 and 96 hour checks, the solid returned at an even greater amount. It is unclear what the what substance was, but it was likely not caffeine because the other concentration of 1 mg/ml of pure caffeine had no white solid fall out of solution. One potential explanation for this solid involves the various other chemicals in the 5-Hour Energy. Perhaps, one of those chemicals broke down after prolonged exposure to oxygen and light, both of which were not controlled in this experiment which formed the visible solid. Another possibility is that one of those chemicals could have reacted with one from the Instant

Ocean causing a product to form in the solution. Finally, the solutions were added to the wells at the same temperature as the refrigerator, before being placed in a warm incubator. These warm temperatures may have hastened any decomposition or reaction of the chemicals.

A mistake was made in the recording of the data and statistical analysis. All wells of treatment were totaled together each day of observation rather than number alive per well. This was done for data collection convenience. However, it means that an average could not be calculated for each treatment and therefore it is impossible to calculate a p-value or standard error of the mean. This error means that it is impossible to determine if this data is statistically significant or not. If this experiment were to be repeated, it would be crucial to record the number living in each individual well for each concentration rather than adding them into one lump sum. Additionally, if this were to be repeated, a much larger sample size would be needed. The amount of time and resources was limiting for a large scale research project.

Conclusion

In conclusion, the results of this experiment can serve as a warning to the human population, but especially pregnant mothers. All groups exposed to any form of caffeine solution on the first day experienced higher levels of embryo death when compared to the negative control. This could apply to pregnant mothers showing that the best idea is to avoid caffeine completely. That being said, the positive control group did not experience nearly as many losses despite equal caffeine concentration. This likely means that some other component works with the caffeine in the 5-Hour Energy which made it much more harmful. Applying this to humans, if a pregnant woman is choosing to consume caffeine, 5-Hour Energy may not be the best choice, especially when so many alternatives exist. The results of the pilot experiment also show a possible connection between age and exposure to caffeine. It appears that if the embryo is older and a bit more developed, it was not affected as much by the substance as those embryos at a very young age.

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