Perpetual Exposure to Varying Concentrations of Caffeine and Its Effects on the Development and Well-Being of Zebrafish Embryos

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Abstract
This experiment was conducted to expand upon prior research on the the effects of caffeine on development of human embryos via testing on zebrafish(*Danio Rerio*) embryos. The zebrafish embryos were placed into a control solution, and three varying concentrations of caffeine. The embryos were observed every 24 hours, from 0 hours post-fertilization, to 168 hours post-fertilization. Some notable results included a spinal deformity, hyperactivity, and a death of all embryos in one concentration.

Introduction
Caffeine(trimethylxanthine) has become a regular occurrence in the status quo, being found everywhere from a cup of coffee to a can of soft drink. In moderation, caffeine has minimal effects on the developed human body, however it has untold effects on developing humans. Human embryos do not have the metabolism to process caffeine, and "caffeine can lead to birth defects and miscarriages"[1]. Despite the facts of the matter, not much research had been conducted to study these effects. The purpose of this experiment was to verify the data of previous findings, by testing the effects that specific concentrations of caffeine had on zebrafish(*Danio Rerio)* embryos. The embryos were exposed to the varying concentrations of caffeine, and the results were compiled. The zebrafish originated in and around the freshwater Ganges river in India. They primarily consume smaller organisms, and are eaten by larger fish, birds, and mammals. From the 1970s onward, the zebrafish has been used for developmental and genetic experiments because their eggs are externally developed and clear in nature, allowing for direct observation of the embryo. Zebrafish are also vertebrates, with a similar physiology to humans, allowing for apparent connections. Their quick developmental span also means that observations and results can be found quickly and cohesively. Zebrafish are also easy to care for, making the experiment easier to conduct.

Methods and Materials
Materials

- Zebrafish Embryos, 40, fertilized
- 0.05 mg/mL solution of caffeine
- 0.25 mg/mL solution of caffeine
- 1.0 mg/mL solution of caffeine
- Embryo media or instant ocean
- One well plate, with at least four columns
- One well plate cover
- One roll of masking tape
- One Sharpie
- Small bore pipettes
- Wide bore pipettes
- One beaker for dead embryos and wastewater
- Incubator, set between 26 and 28 degrees celsius
- One stereoscope
- One microscope

[1] "Caffeine Intake During Pregnancy." *American Pregnancy Association*. N.p., 01 Sept. 2016. Web. 10 Mar. 2017.

- One slide, bowed in the middle

Methods

Using a wide bore pipette, all four wells, A1, A2, A3, A4, were filled with 1 mL of embryo media. Using a separate wide bore pipette, A2 was filled with 1 mL of the 0.05 mg/mL Caffeine solution. A3 was filled with 1 mL of the 0.25 mg/mL Caffeine solution with different wide bore pipette. A4, using a final wide bore pipette, was filled with 1 mL of the 1.0 mg/mL Caffeine solution. Ten embryos were placed in each well using the small bore pipette, one per well to avoid contamination. Daily, excluding Saturday and Sunday due to lab inaccessibility, the number of embryos in each well was counted with the stereoscope and was recorded on the data sheets. The same method was used for embryos hatched. Dead embryos were discarded into the waste beaker. The wells were emptied daily using the small bore pipette, carefully to avoid mistakenly intaking an embryo. The wastewater was emptied into the waste beaker, using a different pipette per well. In order for the wells to be refilled, the same aforementioned steps were taken. Observations of the embryos were made using the observation slide, the embryos transferred with a different small bore pipette. At this point, pictures, using an iPhone 6, were taken (Fig. 1-3). All embryos were returned to their respective wells, and the plate returned to the incubator at 26-28 degrees celsius. At the conclusion of day 8 observations, the embryos were euthanized via freezing.

Results

It was hypothesized that the embryos affected by caffeine would hatch later, have stunted growth, be more active, and would be at a greater risk for mutations. Ten embryos were placed in each, separate well. The control was only submerged in embryo media, and the rest in consecutive levels of concentration, 0.05 mg/mL, 0.25 mg/mL, and 1.0 mg/mL. These levels had remained constant throughout, along with the incubation temperature. The only changes that had been made were the number of fish in each well as they died, and the number of embryos hatched, coupled with their behaviors and appearances.

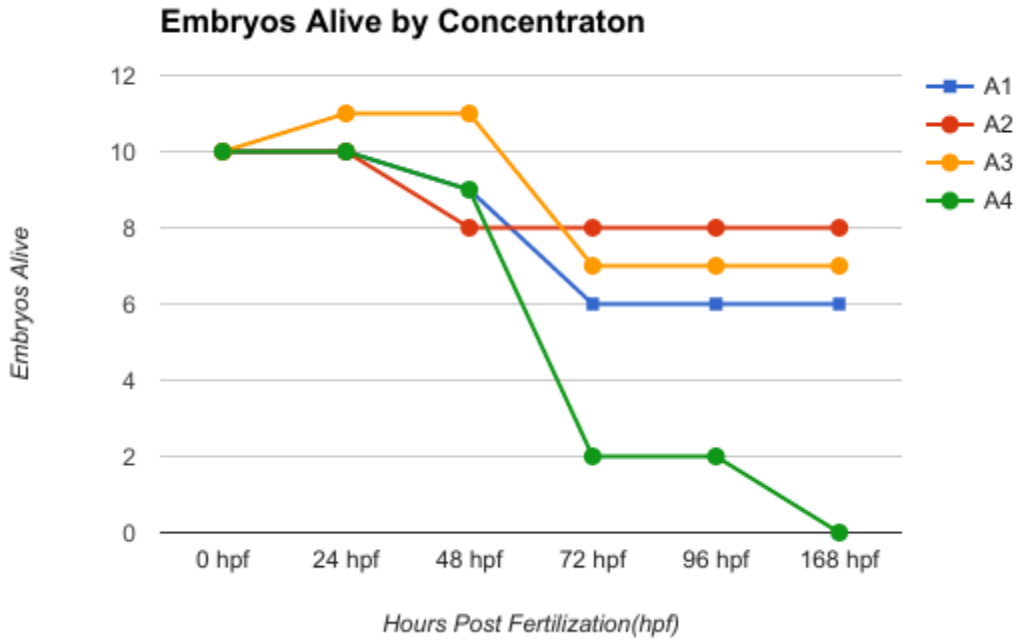
The embryos affected with the 0.05 mg/mL and 0.25 mg/mL concentrations did not hatch any considerable amount of time after the control embryos, but the embryos in the 1.0 mg/mL concentration did not hatch at any point during observations, and all were deceased by day 8. Despite the similar hatching times, the fish in the control seemed notably less active than those in the 0.05 and 0.25 mg/mL concentrations. The control fish tended to sit around when possible, and only really moved small amounts when disturbed by a pipette. The fish in the latter concentrations however also liked to sit around, but when disturbed, would swim around the well, or in the water bubble on the slide for nearly 30 seconds before finally settling down again. Upon the fish's hatching, there was only one discernible mutation in a fish. One fish in the 0.25 mg/mL concentration had a deformed spine, and a bent over tail tip (Fig. 3). The coloration of the fish appeared to be consistent with the control, and the sizes of the fish did not vary beyond what would normally have been expected.

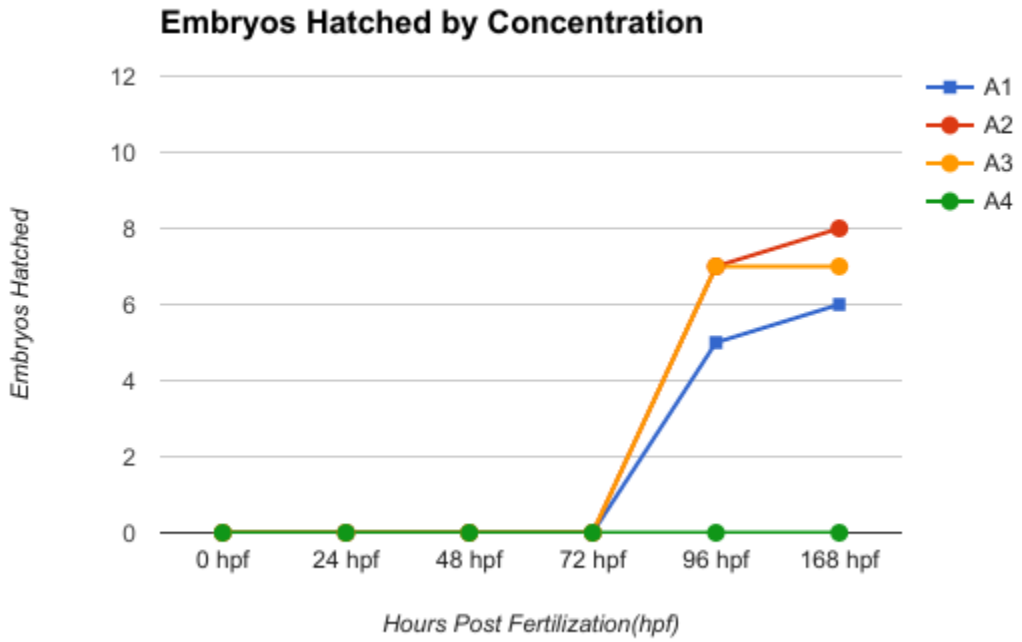
The results did not support the initial hypothesis, as there was no to little difference in the hatching time, and there was only one mutation in the 40 embryos. The fish afflicted by the caffeine were more active, however the other set criteria were not met to a full enough degree to be considered to support the hypothesis. The zebrafish were an excellent model on the physical development of embryos, however we could not measure cognition, mental disabilities, social interactiveness, heart rate disruption, or sleep cycle disruption that caffeine has been linked to in prior studies[2].

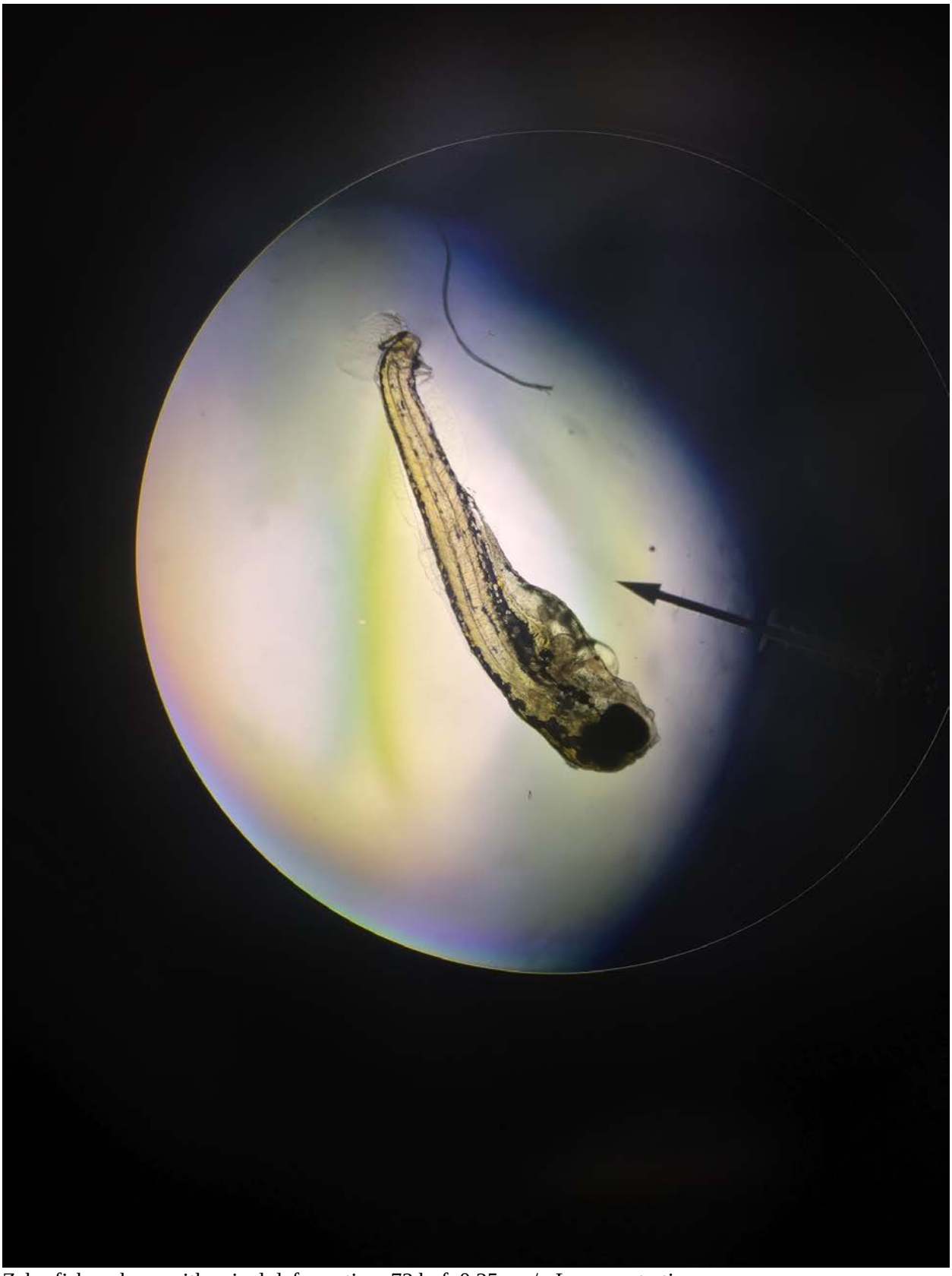
Discussion

Some of the results that should be noted include the hyperactivity of fish in the caffeine solutions the physical deformity of the fish in well A3, and the eventual death of all embryos in well A4. The cause of the deformation could have been a direct result of disturbances by the experimenters, however this is unlikely because only one fish was afflicted, despite nearly identical amounts of necessary disturbance(e.g., water replacement, slide analysis, etc.) in all wells. Those disturbances are also unlikely to have caused the hyperactivity, as that was found in all hatched fish in only caffeine concentrations, not the control. Evidently, the presence of strong concentrations of caffeine in embryonic development can have adverse effects on the embryo, as the embryos in the 1.0 mg/mL concentration never hatched, and all eventually died before euthanization. Excessive activity should also be noted as a significant effect of caffeine in development. There were not enough deformities in the sample size to conclude anything about caffeine being linked to physical deformation.

In whole, the experiment was not conclusive of other studies in the matter, and further research should be conducted on the effects caffeine can have on young children, adolescents, and adults. Due to our inability to measure any sort of cognitive function, or lack thereof, further tests should be run with an emphasis on those areas. The results from such a study would provide a greater insight into the effects stimulants can have on the development of embryos and babies.


Embryos Alive by Concentraton
12
10
8
6
4
2
0
Embryos Alive
0 hpf
24 hpf
48 hpf
72 hpf
96 hpf
168 hpf
Hours Post Fertilization(hpf)
A1
A2
A3
A4


Embryos Hatched by Concentration
12
10
8
6
4
2
0
Embryos Hatched
0 hpf
24 hpf
48 hpf
72 hpf
96 hpf
168 hpf
Hours Post Fertilization(hpf)
A1
A2
A3
A4



Zebrafish embryo with spinal deformation, 72 hpf, 0.25 mg/mL concentration