The Effects of Moderate Chronic Embryonic Caffeine Exposure on Zebrafish (*Danio rerio*) Neurodevelopment

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The Hypothalamic-pituitary-adrenal (HPA) axis is the primary mechanism for the activation and deactivation of the fight-or-flight response, also referred to as the sympathetic nervous system (SNS). This mechanism begins with the recognition of an excitatory stimulus by the hypothalamus, the homeostasis regulator. The hypothalamus sends a signal to the pituitary gland, the hormone regulator, telling it to send a signal to the adrenal glands to release cortisol, the stress hormone, into the bloodstream. Once in the bloodstream, cortisol produces the effects of the SNS, such as elevated heart rate and blood pressure, dilated pupils, enhanced focus, etc. (Smith & Vale, 2006) (Murray & Cheney, 1982)

SNS activation continues until the concentration of cortisol in the blood reaches a peak set-point, at which point, the hypothalamus signals release of adenosine, a nervous system suppressant, that acts on receptors in the hippocampus (Murray & Cheney, 1982). The hippocampus sends a signal to the hypothalamus, telling it to stop releasing cortisol, thereby deactivating the SNS. If cortisol exposure is prolonged, it is actually harmful to these hippocampal cells and can lead to hippocampal neuron death. This decreased hippocampal volume may have more difficulty and take longer to deactivate the SNS in the future (Soellner, 2009).

Artificial SNS activation, such as with psychostimulants like caffeine, can lead to high concentrations of cortisol in the brain. Caffeine is readily received by receptor cells and blocks the adenosine receptor sites, making it harder for adenosine to reach the hippocampus, which prevents the hippocampus from sending its message to the hypothalamus. This will cause cortisol levels to continually increase past the set point. This exposes the hippocampus to higher concentrations of cortisol, which can potentially be harmful to the hippocampus and the HPA axis. (Soellner, 2009)

Caffeine is the most accessible and most consumed psychostimulant, being found in carbonated beverages, coffee, workout supplements, alcoholic beverages, and some foods. Caffeine is a nonpolar, lipophilic teratogen that readily and rapidly crosses the placenta from the mother to the embryo (Reynolds & Knott, 1989). The normal half-life of caffeine is 2-4 hours in adults (Bonati et al., 1984) and 10-20 hours in pregnant women (Aldridge et al., 1981). The placenta does not have the necessary enzymes to transport caffeine out of the amniotic sac back into the mother’s bloodstream, so the half-life of caffeine is greatly increased and ranges from 50-100 hours (Arnaud, 1993), meaning that the embryo has the potential of being exposed to high levels of caffeine for the duration of the pregnancy. Caffeine is thought to be safe for consumption by everyone, however, this availability may have consequences in prenatal neurodevelopment.

In recent years, zebrafish (*Danio rerio*) have become useful as an animal model for toxicological and pharmacological research (Avdesh et al., 2012). Zebrafish eggs, embryos, and adults are primarily transparent, which allows for research into the effects of various substances on internal biological systems with reduced need for dissection of each subject after treatment. Caffeine has been shown to produce anxiety-like and depressive-like symptoms, such as avoidance of bright light and open spaces, reduced locomotion, and concentration of skin pigment (which happens when their SNS is activated), as well as a chronic increase in cortisol concentration in adult zebrafish (Maximino et al., 2011) similar to the effects of caffeine in the adult human brain. In zebrafish, adenosine receptor cells have been identified as early as 24 hours post-fertilization (hpf) and concentrated in similar regions of the brain as humans (Boehmler et al., 2009). Because these areas are similar to that in humans and show adenosine receptor accumulation, it can be hypothesized that the higher the concentration of caffeine and the longer the duration that the zebrafish are exposed to caffeine prenatally, the more severe the anxiety-like and depressive-like symptoms will present in adulthood.

Materials and Methods

Zebrafish Care and Maintenance

Four male and four female zebrafish were housed in breeding pairs in a top-siphoning zebrafish tank system at 28ºC. Pairs were on a 12h light/dark schedule, with the onset of light at 0800h. They were fed crushed, autoclaved blood worms once a day, at 0930h, until satiated. Breeding and egg-collecting were performed using an in-tank breeder, placed on the previous night. Egg collection took place Tuesday-Thursday of each week.

Preparation of Egg Water and Embryo Medium

A stock solution of 40g Instant Ocean in 1L DI water was prepared prior to experimentation. During experimentation, a solution of 1.5mL stock solution in 1L DI water was prepared for the application of treatment.

The following Hank's solution was prepared and used as an embryo medium: 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4, 4.2 mM NaHCO3. (Westerfield, 2007)

Egg Collection and Preparation

Eggs were transferred from the in-tank breeders to square, gridded petri dishes using a micropipette with a trimmed tip, to avoid damaging eggs. Using Carnegie's Stages of Development (Kimmel et al., 1995), eggs were observed under a dissecting scope, classified, based on age, and transferred to a 12-well dish for treatment. Each egg was given a separate well for treatment.

Application of Treatment

According to the Mayo Clinic (2017), a single cup of coffee has 95-165mg of caffeine, Assuming that all of that caffeine reaches the bloodstream, the concentration of a single cup's caffeine in the average human's blood (~5.0 L of blood) would be 26mg/L. To account for variability in the average number of cups of coffee consumed in a day, the concentrations for one cup (26mg/L), three cups (78mg/L), and six cups (156mg/L) were investigated. These concentrations were prepared by dissolving the respective amount of caffeine for each concentration into a prepared solution of autoclaved egg water Because zebrafish hatch at approximately 48 hours post-fertilization (hpf), the durations of 12h, 24h, and 48h were selected to simulate chronic fetal exposure during pregnancy. At the completion of caffeine exposure, the treatment solution was siphoned out of the wells and replaced with fresh embryo medium. After one week, fries were transferred from the 12-well dish to a square, gridded petri dish for the remainder of the study. Fish of the same condition and of the same dish were placed into the same petri dish.

Euthanasia Technique

At the conclusion of the study, all Zebrafish were humanely euthanized by submersion in near freezing water until 15 minutes after last gill cessation. Euthanasia was carried out using IACUC-approved techniques.

Measurements

Heart Rate was measured using a dissecting microscope and a timer. The subject was placed in the field of view of the microscope lens and the number of heart beats in 15 seconds was recorded and multiplied by four to yield an estimate for the heart rate. Length was measured using a dissecting scope and a sheet of quad-ruled, 1 mm x1 mm graph paper placed underneath the petri dish holding the fish. The specified subject was placed within the field of view of the dissecting scope lens and the length of the fish was compared to the graph paper. Avoidance of light was measured by placing a petri dish onto the stage of a dissecting scope and placing a piece of duct tape with a 1mm-hole punched through the center over the top light of the dissecting scope. A camcorder was pointed at the stage and recorded the petri dish for two minutes and was repeated for each petri dish. All data was analyzed using a one-way analysis of variance, using IBM SPSS. At the conclusion of the study, all zebrafish were humanely euthanized by submersion in near freezing water until 15 minutes after last gill cessation.

Results

Changes in caffeine concentration and duration did not show a significant relationship to changes in heart rate in zebrafish, $F(3, 5) = 0.238$, N.S.

Changes in caffeine concentration and duration did not show a significant relationship to changes in length in zebrafish, $F(3, 38) = 0.986$, N.S.

Changes in caffeine concentration and duration did not show a significant relationship to changes in the number of times that the fish crossed the line into the light.

Discussion

During the study, the fish are thought to have become infected with *Pseudoloma neurophilia*, a microsporidia that affects the nervous system and can lead to lethargy, spinal abnormalities, and death (Matthews, 2018) (see Fig. 1). This microsporidium also affects the ovaries, making eggs susceptible to infection. Embryos and fries exposed to *P. neurophilia* are shown to be more susceptible to infection and result in high mortality, compared to adult fish (Ferguson, Watral, Schwindt, & Kent, 2007). In this research, infection was only observable post-mortem, even though behavior can be affected before death. It was observed that all fries died and showed signs of infection by the conclusion of the experiment. During this experiment, two adult breeding fish died (one from suffocation and one by a dorsal lesion), but their deaths did not appear to be related to the infection.

Researchers conclude that the collected eggs were infected with this parasite upon fertilization and remained infected for the duration of their lives. Because the parasite affects behavior before showing signs of infection, none of the data collected can be used for unconfounded hypothesis testing.

A repeated study with more safety and precautionary measures, such as cleaning eggs and better controlling of water purity, should be performed to investigate the effects of embryonic caffeine exposure on neurodevelopment.

Appendix A: Figures

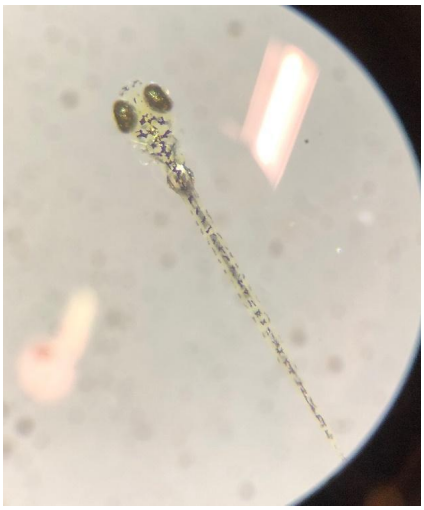

Figure 1: *An Uninfected Fish and a Fish Infected with P. neurophilia*

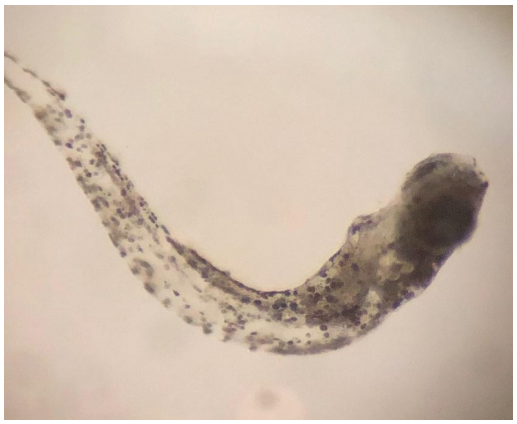

Figure 2a *: An infected Fish and a Fish Infected with P. neurophilia*

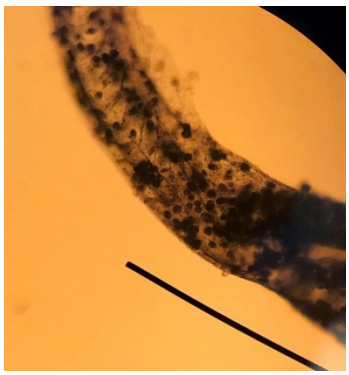

Figure 2b*: An infected Fish and a Fish Infected with P. neurophilia*

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