Assessment of Blood Vessel Area Growth in the Developing Chick Embryo under chronic exposure to 0.5 mL of caffeine

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Introduction

Vasculature formation is critical in the developing embryo. Growth and differentiation of blood vessels are essential in supplying nutrients and oxygen to the forming tissues and organs. It is this vasculature that establishes complex delivery highways used by the embryo during growth. Accordingly, the major function of the vascular system is to exchange materials between blood and tissues (Scanlon & Sanders, 2007).

Blood vessels are an intrinsic part of the growing embryo. Found everywhere, they stimulate growth in precursor organs by supplying nutrients and ridding the body of waste in the process. Specifically, vessels act to deliver oxygen to cells and tissues while removing urea and other waste in the case of the chick embryo. In a study performed by Nehlig and Debry (1994), rodent experiments showed that caffeine can be teratogenic when given in large, single doses. If blood flow is compromised, the life of the organism is in jeopardy. With a reduced amount of nutrients as well as waste build-up, the embryo's organogenesis is impeded. If environmental teratogens such as caffeine are allowed access into the embryo, their toxic effects on development can be irreversible.

In this experiment, my lab partners Sara Mason, Carrie Pingree and I investigated the potentially damaging effects of daily coffee consumption on the developing fetus during pregnancy. Our independent research modeled chronic caffeine exposure in the chick embryo.

According to a publication by the National Institute of Child Health and Human Development, drinking four or five cups of coffee per day while pregnant increases the chance of miscarriage two-fold over pregnant women who drink little to no coffee a day (National Institute of Child Health and Human Development [ICHD], 1999). Also, the American Pregnancy Association states that caffeine is known to cause birth defects, preterm labor, low-weight babies and in some cases, miscarriages (American Pregnancy Association [APA], 2007). However, there is inconclusive evidence regarding the specific effects of caffeine on the cardiovascular health of the fetus if a pregnant mother has moderate, daily exposure to caffeine.

Measurement of the projected area of the blood vessel, that is, the amount of 2-D area seen when the 3-D vessel is projected onto a 2-D screen is an important consideration in assessing the health of the embryo (R. Morris, personal

communication, October 26, 2007). If decreased, the blood vessels cannot transport nutrients as efficiently and the developing tissues of the embryo should suffer.

According to a study done by Hawkins, Hu and Clark (1984), caffeine affects vasodilatation and contraction in the heart of the 24-hour embryo. Specifically, it was reported that cardiovascular function was impeded by increasing myocardial contraction and dilating resistance vessels, such as the vitelline vein.

In our lab, I measured another facet of cardiovascular function in the presence of caffeine using chickens. Chickens are model organisms in the study of development. As amniotes, they undergo vertebrate development common among many species, including Homo sapiens. The animal is readily obtained and the hatching cycle is independent of season (Tyler, 2006). In this study, I tested the hypothesis that `chronic exposure to caffeine would inhibit blood vessel formation as measured by projected area of the vasculature in the 3-10 day explanted chick embryo (R. Morris, personal communication, October 26, 2007).` My lab partners and I explanted chick embryos and subjected half of the group (experimental) to 0.5mL of caffeine on a daily basis. Using the Wheaton Imaging Center for Undergraduate Collaboration (ICUC), I captured images of both anterior and posterior developing vitelline veins in each embryo and measured projected area using *Image J* software between one and eight days of incubation.

Materials & Methods

Materials

Unfertilized, store-bought eggs, forceps and an unsterile weigh boat were used to practice explanting chick embryos.

Sterile forceps, eight sterilized wax-bottomed weigh boat petri dishes, latex gloves, an empty egg crate container, paper towels, Kim wipes, a permanent marker, 70% ethyl alcohol (EtOH), and a waste beaker were used to explant eight 72-hour fertilized chick embryos. A 37 degree Celcius incubator was used to house all successfully explanted embryos.

A P1000 micropipettman, sterile tips, 2 sterile vortex tubes, an analytical balance, 7 mg of Sigma-Aldrich C0750-100G caffeine powder, 35mL of Tyrode's solution, 3.5mL of penicillin/streptomycin solution and latex gloves were used to prepare and deliver injections for control and experimental groups.

A Macintosh computer (Aries) and printer, *BTVPro* Image software, *Image J* software, a Nikon SMZ-660

camera, ruler, and an external light source, all found in the Wheaton Imaging Center for Undergraduate Collaboration (ICUC) were used to collect images and analyze vasculature data for each embryo.

Methods

A modified version of the Armstrong et al. (1994) experimental procedure was used to explant the eight embryos. This explanting method was adapted from the Chick-in-a-boat methods section (McKay, 2007). Prior to explanting, all bench top surfaces were sterilized with 70% ethyl alcohol (EtOH) using paper towels. Rubber gloves were used and sterilized again with 70% EtOH. Fertilized, 72-hour chick eggs were removed from the 37˚ incubator and placed in an empty egg shell crate for transport. One sterilized forceps and eight sterilized, wax-bottomed weigh boats were also obtained. Using EtOH and a few Kim wipes, each egg was sterilized, and air-dried after being placed wide end down in the crate. Like the Chick-in-a-boat study, we did not use Betadine solution as indicated in the Armstrong et al. experimental procedure. Once completely dry, each egg was removed from the crate using sterilized gloves and held over a sterile waste beaker. Holding the egg narrow end down, a small hole was cracked in the wide end using a sterile forceps. Here, the air pocket was observed. The tips of the forceps were used to chip away at the egg shell and create a large enough hole for the embryo to exit the shell. Once a smooth hole was made using careful forceps technique, the discard beaker was replaced by a sterile, labeled weighing boat and not a Dixie cup, as indicated in the Armstrong et al. (1994) procedure. Carefully, the egg was rotated 180˚ and held low over the weigh boat. Using the tip of the forceps, a small hole was punctured in the narrow end of the egg to release the embryo. If the embryo did not exit the shell, a small nick was created in the membrane holding the embryo in the wide end of the egg. Some albumin leaked out of the egg. *Patience* was a key factor in this procedure! Slowly, the embryo and its yolk sac exited the shell and onto the sterile weigh boat. Vasculature and in some cases, a heartbeat were observed in the explanted embryo. All explanted embryos were assessed for viability before being placed in the 37˚ incubator.

Chronic Exposure Injections

Approximately 7 milligrams of Sigma-Aldrich C0750-100G caffeine powder was massed out using the ‘tap’ method on an analytical balance. The powder was then dissolved in 35mL of Tyrode’s solution giving an approximate concentration of 0.21mg/mL. Once the solution was prepared and labeled in a sterile vortex tube, our group also obtained 35mL of plain Tyrode’s solution and labeled it in a sterile tube. Both solutions were refrigerated. A P1000 micropipettman was obtained from Professor Morris’ office and used to deliver volumes of pen/strep, caffeine and Tyrode’s to each group. Once all embryos were explanted, they were labeled as “Control” or “Experimental” and

numbered from 1-7. All embryos were injected with 0.5mL of the cocktail solution (pen/strep) containing penicillin (1u/mL) and streptomycin (0.01mg/mL) to guard against bacterial infection. The tip was replaced on the pipettman prior to Tyrode's injection. Three of seven explanted embryos were labeled "Control" and were injected only with 0.5mL of Tyrode's solution using sterile technique. The controls were a vital part of the experiment used to reduce the number of variables. They acted as a standard to compare growth and development in the experimental caffeine embryos. Without the controls, the results of our experiment would be inconclusive without reference to normal chick development. The tip was changed before injecting the remaining four embryos with 0.5mL of caffeine solution (0.21mg/mL). All labeled solutions were returned to the refrigerator and embryos were replaced in the 37 degree incubator.

Data Collection & Analysis

All embryos were monitored over an 8-day incubation window. Three different trials were performed to increase the n value. Each trial lasted approximately one week and embryos were injected on a daily basis. Each daily injection and data collection lasted about 15 to 20 minutes per embryo. Once all embryos were injected using sterile techniques, the solutions were again returned to the refrigerator prior to data collection. One at a time, each embryo was transported into the ICUC being careful not to contaminate the outside lip of the weigh boat or petri dish with albumin. In the Imaging Center, the dissecting stereomicroscope was used along with an external light source to focus the vitelline veins on magnification objective 2. The "Aries" Macintosh computer was used to bring up *BTVPro Image* software to collect data by generating a live image of the embryo on the desktop. Using the SMZ-660 Nikon camera, an image of each vitelline vein was captured and printed using *BTVPro*. Once all images were collected and appropriately labeled, *Image J* software was used to analyze the data. The "measure" feature was selected to estimate the approximate projected area of each vein in pixels squared. This value was recorded in the notebook and the image was saved and labeled on the desktop. For ease of future conversion calculations, an image of a ruler was captured on magnification objective 2 to determine the approximate number of pixels in one millimeter. Once all data was collected, the embryo was returned to the incubator and inspected to be sure no albumin was transferred onto the cover of the dish during transport. This process was followed for each embryo for one to eight days depending on the lifespan of the chick.

Results

The following plots were generated to analyze vitelline vein growth under normal development and chronic

caffeine exposure.

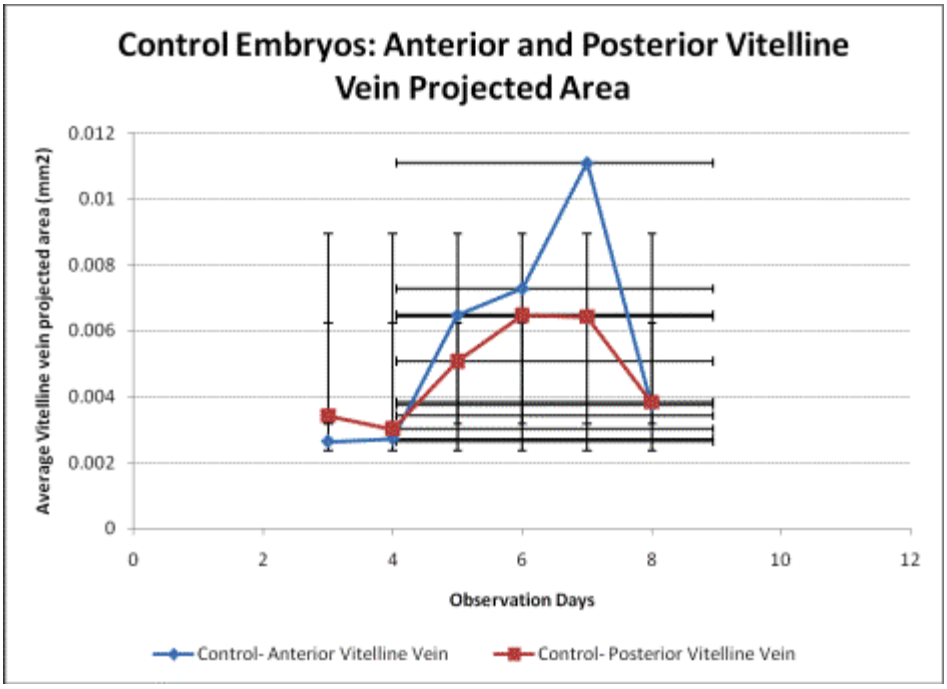

Figure 1: Anterior and Posterior Average Projected area of Vitelline Vein in Control Chick embryos over 6 observation days, n = 2 chick embryos in Trial 1.

According to Figure 1, there is a correlation between increased vein area and observation days in both anterior and posterior portions of each embryo. This trend is observed until day seven, when both veins taper off in projected area. The decrease in area may be attributed to impending death of the organism, and not necessarily environmental factors or contaminants.

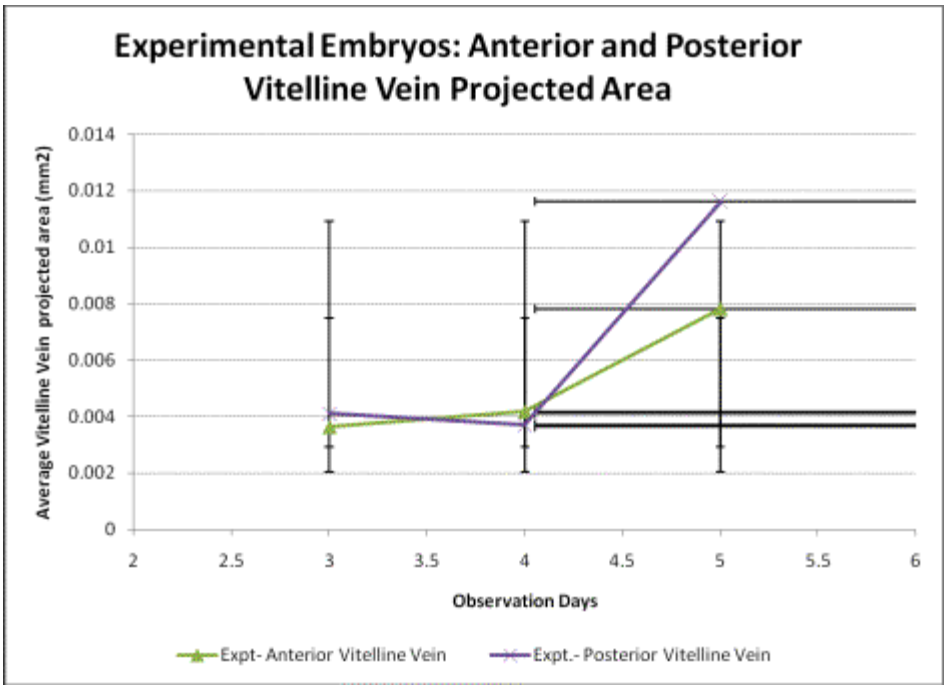

Figure 2: Anterior and Posterior Vitelline Vein projected area in Experimental chick embryos over 3 observation days, n= 5 chick embryos in Trials 1 and 3.

Only 3 days of experimental embryo data were collected. In Figure 2, there is again a positive relationship between increasing observation days and projected area. Unfortunately, all embryos had died by the third day so that this trend could not be followed as it was in the Control group. Interestingly, both Controls in Figure 1 and Experimentals in Figure 2 show larger growth in the anterior veins.

Figures 3 and 4 were generated to show basic relationships of vitelline vein projected area and incubation period between the Control and Experimental groups. Averages were taken combining both anterior and posterior veins for all data to make for more compelling evidence of impeded blood vessel development in the presence of caffeine.

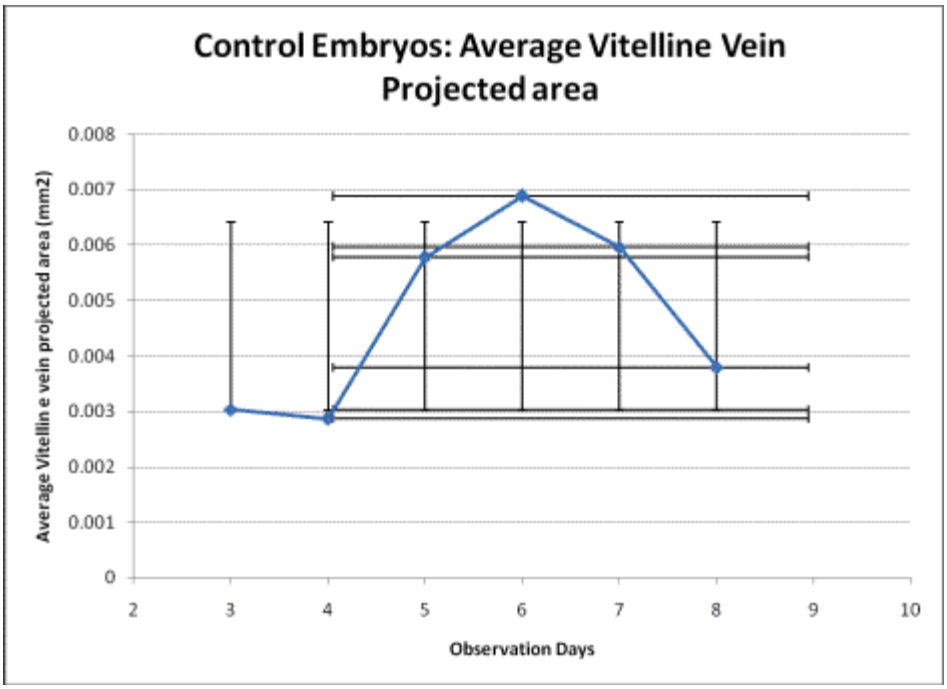

Figure 3: Average Vitelline vein projected area for Control Embryos over 6 incubation days, n=2 chick embryos in Trial 1.

Figure 3 was created to use as a standard reference for normal development of explanted chick embryos. As expected, increased incubation days led to larger projected blood vessel area. However, it is important to note the sharp decrease in vitelline vein area after six days of incubation. The reduced vasculature observed in dying embryos may account for this trend.

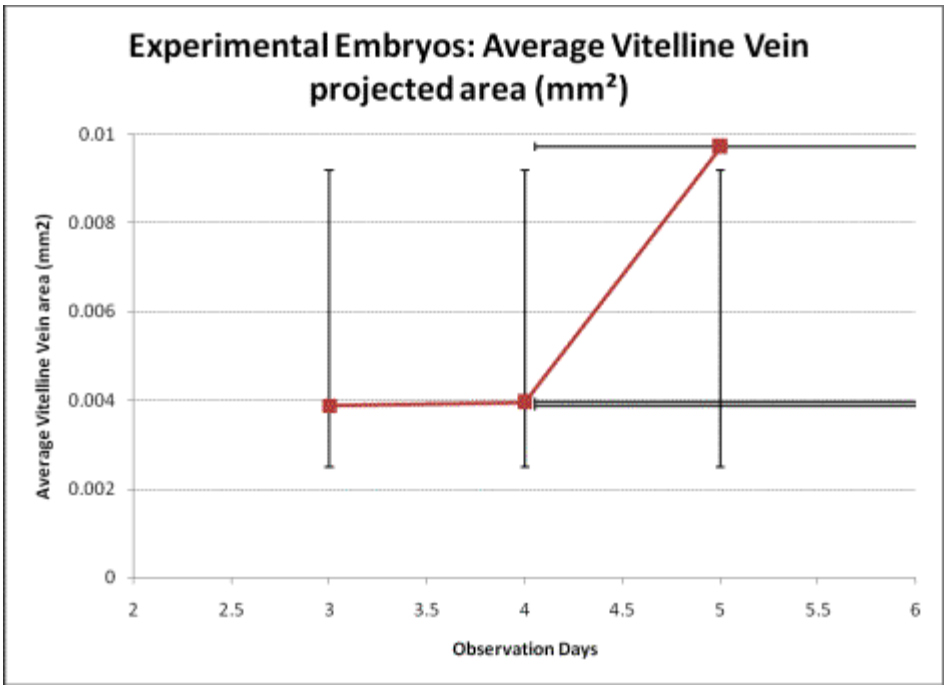

Figure 4: Average Vitelline vein projected area for Experimental Embryos over 3 incubation days, n=5 chick embryos in Trials 1 and 3.

Figure 4 shows little activity in blood vessel development between the third and fourth day of incubation. The average area increases dramatically between days four and five. Over the course of three trials, there were more chicks to observe past the third day of observation in the experimental group (n=5 chick embryos) as opposed to the Control group (n= 2 chick embryos).

The following images demonstrate typical views of anterior and posterior vitelline veins of the chick in five days of development.

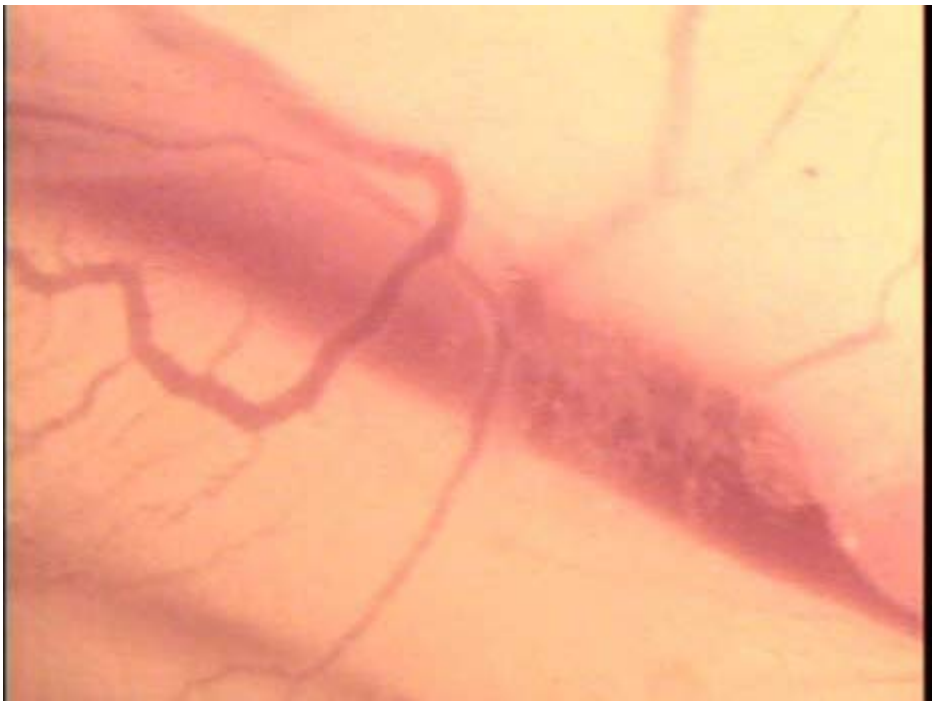

Figure 5: Anterior Vitelline vein of Control explanted embryo at five days of development.

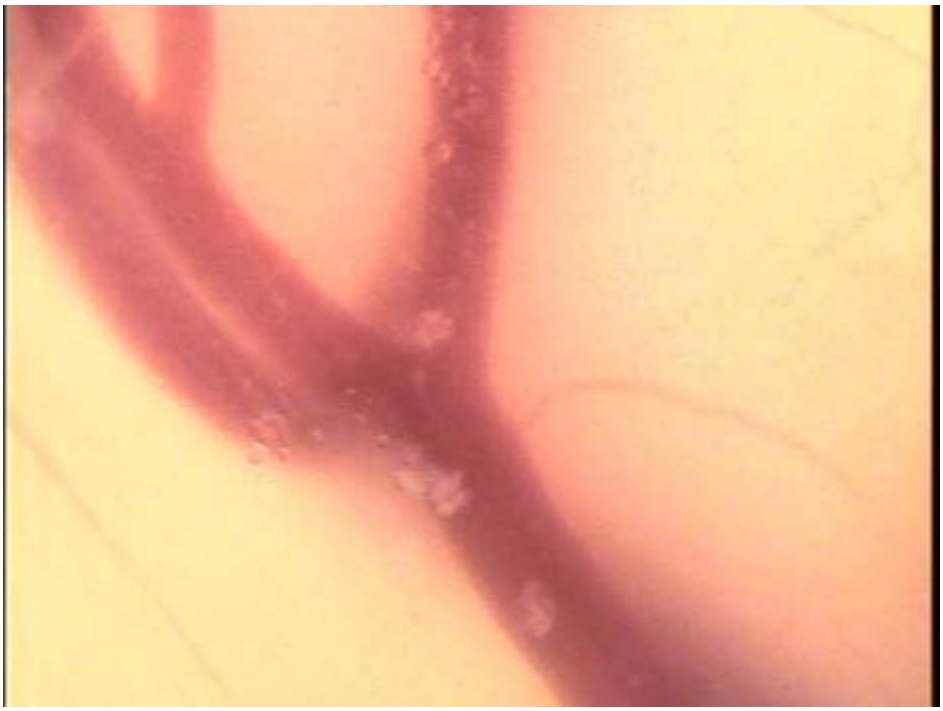

Figure 6: Posterior Vitelline vein of Experimental explanted chick embryo at five days of development.

Discussion

There is inconclusive evidence from the data to support the hypothesis that chronic caffeine exposure leads to reduced blood vessel area. According to Figures 3 and 4, there is no indication that over eight days of incubation, the experimentals have reduced blood vessel formation compared to the controls. In fact, Figure 4 shows that the average maximum blood vessel projected area for experimental was larger at almost $0.01mm^2$ than $0.007mm^2$ for controls. The

anterior vitelline veins appeared to grow faster than the posterior veins but this result is not directly related to the original hypothesis, suggesting that further research be conducted in assessing vein location in development. Based on these results, the hypothesis that chronic caffeine exposure reduces blood vessel formation must be refuted pending further experimentation.

Some of the data collected in the three trial weeks of experimentation were omitted from this report. Data used to generate Figures 1-4 were taken from the first and third trials. The second trial data were not used in this report because the embryos had been kept at 19 degrees Celsius for a week longer than all other embryos and this appeared to compromise survivability and caused stunted development at 72 hours of incubation. The data from this trial were not comparable to trials 1 and 3. Using only two of three weeks worth of data reduced the n value, but was necessary to avoid analysis of external variables besides caffeine exposure.

There were significant errors committed in this independent research. While all measurements were taken by one individual, the consistency of the location of blood vessel is questionable. The approach of data collection was to find comparable areas in the anterior and posterior regions of the blood vessel, however the raw data suggests that these two values did in some cases, differ by more than a half millimeter. In addition, the *Image J* tool did not allow exact measurement to keep all data standardized. One attempt to reconcile this issue was to measure a ruler under magnification objective of 2 to determine the number of pixels in one millimeter and convert all data to millimeters squared. Also, every measurement was taken on the same computer “Aries” and the SMZ660 Nikon camera using the 2 magnification objective. It is clear from the printed images found in the notebook that the blood vessel areas are slightly different in each embryo.

Additional errors in this experiment prevented my lab partners and I from reaching conclusive results about chronic caffeine exposure and vasculature development (R. Morris, personal communication, November 26, 2007). Embryos were not injected with caffeine or Tyrode’s solution at the same time each day. This may have led to inconsistent data caused by changes in physiological response to the stimulant depending on the time of day. If chicks were left in the ICUC for too long, this would have jeopardized their survivability which may have caused such small observation windows in the majority of embryos. Finally, transporting embryos between the wet lab and ICUC created considerable disturbance to the yolk and albumin of the embryo. I found that upon returning the embryo to the incubator at the conclusion of a measurement session, there was a sticky film of yolk on the lip of the weigh boat. Before putting the chick in the incubator, I would clean the lip and the lid of the Petri dish with 70% EtOH to safeguard against future bacterial infections. Despite these efforts, I would often remove the chick from the incubator at the beginning of a measurement session and find the lid suctioned to the bottom with another layer of dried yolk.

There are many simple, feasible ways of refining this independent work. First, one could be sure to inject and measure the embryos at the exact same time of day. All embryos could be kept in a location closer to the ICUC to avoid excessive transport. Gloves could be worn to decrease the embryos exposure to atmospheric contaminants. The stereomicroscopes could be kept in the Tissue Culture room to keep a constant fan blowing over the exposed embryos again to maintain sterility.

Future experiments include testing a much larger pool of chick embryos. The major difference between this small project and published papers is the n-value and using a larger population of chicks to study gives more credibility to findings in the scientific community (R. Morris, personal communication, November 14, 2007). It might be interesting to conduct the same experiment at higher concentrations of caffeine and to test three groups exposed to high, low and zero levels of stimulant. It would be helpful to keep the chicks in two separate incubators to avoid excessive opening and closing of the large incubator door. Since all embryos were kept in one incubator, the door would have been opened at least twelve times per day as each of six groups opened and closed the doors to measure the embryos.

Published research in the field supports the idea that caffeine can be detrimental to a human fetus if subjected to high concentrations. However, most pregnant women hopefully do not consume such dangerous levels of caffeine during pregnancy (eight to ten cups per day).

Ultimately, the goals of this experiment were achieved. My lab partners and I were given the responsibility of designing a feasible research project and caring for living organisms over the length of a three week experiment. The difficulties of creating solutions, maintaining sterility and successfully explanting living creatures were realized. In data analysis, I was given the opportunity to navigate new software, namely *Image J*. The research has many ways to be improved, but the work of proposing, conducting and analyzing research was an insightful experience no text book could provide.

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