The effect of chronic caffeine exposure on heart strength and rate of developing chick embryos

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Independent Research Project Report
Bio254- Developmental Biology
November 28, 2007

1. Introduction:

The effects of caffeine consumed by pregnant women on the development of their fetus's heart strength and rate are important topics to research. In the United States, caffeine is often studied because it is found in many of the beverages that much of the population consumes on a daily basis, such as coffee, tea, and carbonated soft drinks. Caffeine can have some pharmacological effects on the consumer. A lot of research is focused particularly on caffeine consumption of children and pregnant women because the adverse health effects may be even more detrimental (Knight et al., 2004). Some studies have found that caffeine consumption during pregnancy is associated with an increased risk of spontaneous abortion, low birth weight, and reduced heart rate for newborns (Tsubouchi et al., 2006). Although there has not been any conclusive evidence supporting the claim that caffeine during pregnancy has detrimental effects on development, scientists and doctors agree that any drug that can cross the placenta to the embryo is potentially hazardous (Lee et. al, 1982). Therefore, more research must be done to gather more data for conclusive evidence. In this study, the cardiovascular effects, specifically heart strength and rate, of chronic caffeine exposure on a developing embryo will be studied.

In order to study the cardiovascular effects on an embryo from caffeine, an animal model must be used. To model human development, chicks, rats, and mice are often used because they are easily obtainable, the results and conclusions can be better translated back to humans because their early development is similar to that of mammals (Zajac & Abel, 1992 and Lee et. al, 1982). 72-hour chicks are going to be used in this experiment because the heart is visible and can be more easily studied. The hypotheses that will be tested are that heart strength in the developing chicks, as measured by heart size during each contraction and expansion of a beat, will decrease when given a daily dose of 0.2 mg/ml caffeine solution, and that the pulse in these chicks will increase when given the daily dose of the caffeine solution compared to the control chicks.

To test this hypothesis, 72-hour chick embryos will be explanted from their shells into weigh boats and Petri dishes that will be incubated. All of the embryos, except the controls, will be given a daily dose of 0.5mL of 0.2mg/mL caffeine solution to mimic chronic caffeine consumption (Crawford & Park, 2004). The experiment will start on the day

the embryos are explanted and will continue until day 10 of development, a week later. Heart strength will be determined by measuring the difference in the size of the heart during its contractions and expansions using time lapse pictures and videos. A large difference will correlate to high heart strength. The results over the 7 day experimental period should yield data that will either support or refute the hypotheses.

2. Materials and Methods:

2.1 Embryo Explanting:

The experiment for each of the three trials begins by collecting materials and sterilizing the lab area. The materials needed are a pair of forceps, Petri dishes lined with blue, plastic weighing boats for each chick being explanted, an empty plastic beaker, and an egg crate to hold the eggs. The lab bench, our hands (or gloves), forceps, Petri dishes, and weighing boats are all sterilized using 70% ethyl alcohol to limit any contamination that may occur when the chick is explanted from its shell. The Petri dishes are also labeled on the edge of the top cover and on the bottom with a permanent marker. Once the area is sterilized and dishes are labeled, the 72-hour developing chick egg being explanted is taken out of the 37°C incubator. It is also sterilized using the 70% ethyl alcohol and is allowed to air dry in the egg crate with its wide end on the bottom.

To begin the explanting process, the egg should be held with the wide egg facing up. The forceps will be used to crack the shell on this end where the air space is located, while being careful not to puncture the shell membrane. Once the wide end has been cracked, the forceps will be used to remove the shell covering the air space, making the edges as smooth as possible. The hole should be about a diameter.

After the shell covering the air space is removed, the egg is held over the weighing boat with the hole on the bottom and the small end up. A small puncture is made on the egg membrane near the hole on the bottom using the tips of the forceps. A small crack is then made on the small end of the shell, which is pointing up. Once the hole is made on the top, the embryo will flow out of the hole on the bottom into the weighing boat. Make sure to hold the egg as close to the Petri dish as possible. Immediately cover the Petri dish and place it in a 37°C incubator making sure not to stack any dishes (Armstrong, 1994).

2.2 Caffeine Spiking:

The living, explanted embryos will be divided into two groups: control and experimental, which will be spiked with a 0.2 mg/mL concentration of a caffeine/Tyrode’s solution (Crawford & Park, 2004). (The caffeine/Tyrode’s solution will be made by adding 7 mg of caffeine powder to 35 mL Tyrode’s solution to make a stock solution. The Tyrode’s solution is sterile, so there won’t be any need to do further sterilization on the solution.) Each embryo will

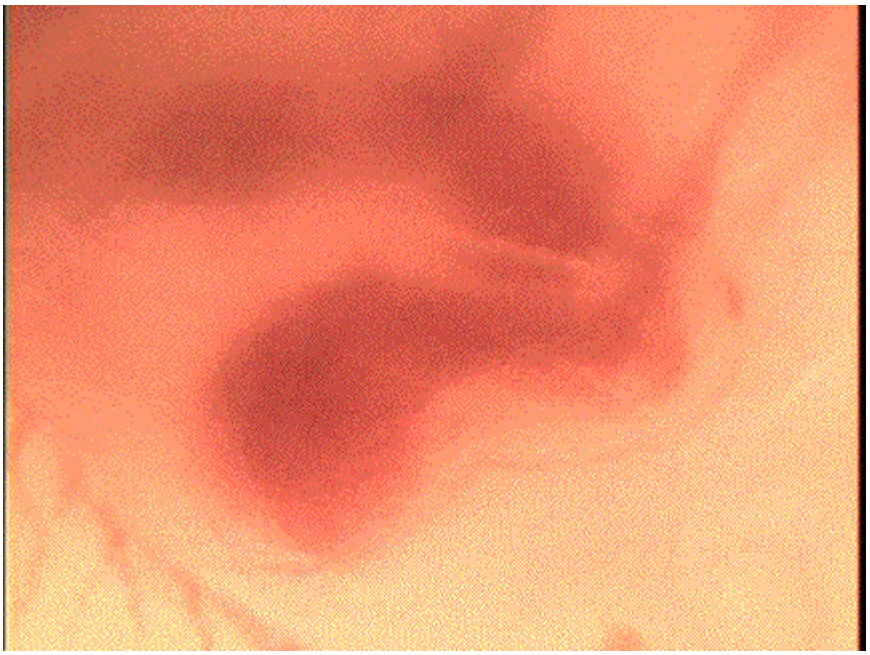
have 0.5 mL of penicillin and streptomycin solution added to it to try to prevent contamination by bacteria. The spiked embryos will have 0.5 mL of the caffeine solution added to it daily, while the controls will have 0.5 mL of plain Tyrode's solution added to it daily in order to maintain a consistent volume in the weighing boats. The controls will allow us to account for any effects the Tyrode's solution will have on the embryos without the caffeine (Armstrong, 1994).

2.3 Measurements:

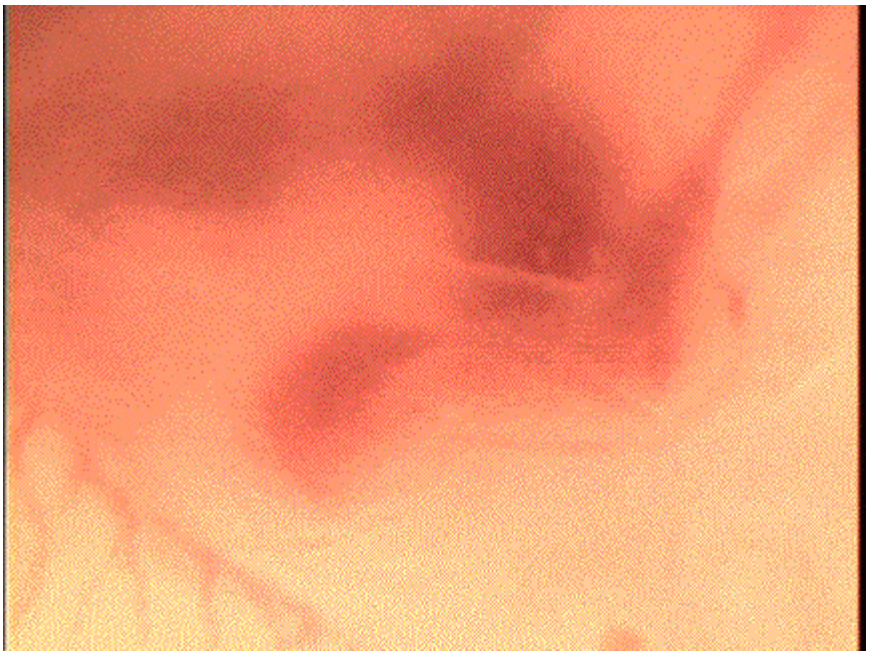
Each day immediately after removing the embryos from the incubator, the caffeine solution or Tyrode's solution will be added to the dishes. The pulse will be measured after the caffeine is added by counting the number of beats in a fifteen second period. This number of beats will be multiplied by four to determine the number of beats per minute. The pulse will be taken every day.

Heart strength will be measured using the dissecting microscope, video cameras, and the BTV Pro computer program. After the pulse is taken, the embryos will be placed on a Nikon SMZ 660 microscope equipped with the video cameras that are attached to a computer with BTV Pro. The focus of the microscope will be specifically on the heart of the embryo at whatever magnification gives the best image. The magnification will be accounted for later so it is critical that it be recorded. The camera will be set to take time-lapse pictures to try to capture the contraction and expansion of the embryo's heart for a minute. To obtain pictures of the expansions and contractions, the videos will be slowed and paused when the heart is at its largest and smallest. A picture of the contraction and expansion will be printed for each embryo.

To measure the heart during the beat, the images used for the expansions and contractions will be exported as jpeg files from the movie. (This can be done by going to File and then clicking Export Frame.) Another computer program, J Image will be used to do the measurements. Once the image is opened in J Image, a tool on the program will allow for a line to be placed on the length to be measured. To find the length, go to the toolbar at the top of the program and click Analyze. A box will appear with the length in pixels. This number is recorded for the measurement. An example of the measurement taken is shown in Pictures 1 and 2 located below.



Picture 1: This is an image of the length measured for the expanded heart of Caf 2, the experimental embryo, on day 3.



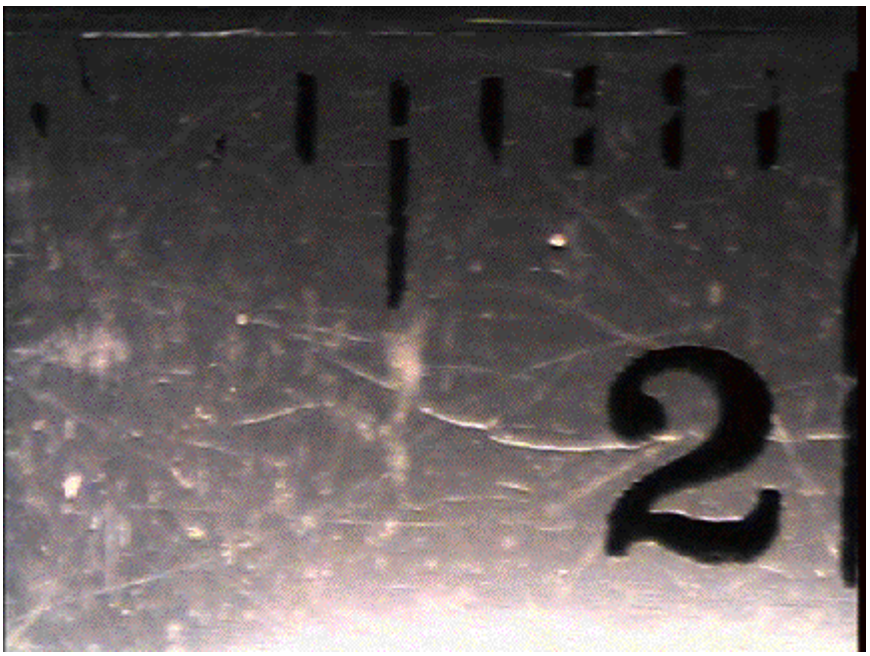
Picture 2: This is an image of the length measured for the contracted heart of Caf 2 on day 3.

There is a clear difference between the measurements of the expanded heart and the contracted heart for the chick.

2.4 Data Analysis:

Once the data is collected, it has to be analyzed. The pulse measurements will be graphed on Excel to see any trends and to compare to the pulse of the caffeinated chicks to the control chicks. The measurements of the heart contracting and expanding will have to be manipulated before it can be graphed. Depending on the magnification of the microscope, the pixels that the contractions and expansions were measured in will have to be converted millimeters so the results are more easily understood. The conversion from pixels to millimeters will have to be done by measuring a

ruler in the magnifications used for the heart videos. Take a picture of the ruler in the magnification, open the image in J Image, and measure the length of a millimeter on the ruler the same way the heart was measured. This will give how many pixels are in one millimeter. An example of the image used for magnification 1 is shown below in Picture 3.



Picture 3: This shows where the measurement was taken to find how many pixels are in a mm so we are able to convert the data to units in mm.

Using the images like the one above, it was found that at magnification 1, 1 mm equals 108.000 pixels, at magnification 2, 1 mm equals 203.002 pixels, and at magnification 5, 1 mm equals 540.015 pixels. These are used as conversion factors to make sure all of the units are in millimeters.

To account for inherent differences in heart size between the embryos, a percentage is taken that will give the percentage of the difference between the expanded heart length and contracted heart length, so the results can be normalized. The higher the percentage, the bigger the difference in the expanded and contracted heart length, which correlates to increased heart strength. A generic example of this calculation is shown below:

100 - (Contracted Heart Length/Expanded Heart Length * 100) = Percentage of the difference between the expanded and contracted heart lengths

These percentages will be graphed on Excel to see any trends in the differences in heart strength between the control and experimental embryos.

3. Results:

Over the course of three trials, fourteen out of twenty-four embryos were successfully explanted from their shells in order to perform this experiment. However, the survivability of the embryos was not as expected. The chart below,

Figure 1, depicts how many embryos were alive each day for each of the three trials.

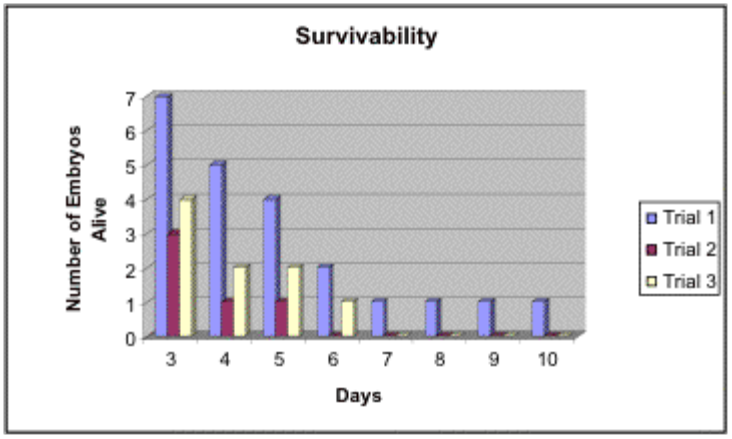


Figure 1: This graph shows how many embryos were alive at each day of the experiment for each trial. All fourteen of the embryos that were successfully explanted were included.

In order to collect quantifiable data to show the differences between pulse and heart rate, at least one control and one experimental have to survive at least three days after being explanted (day 5). From Figure 1 it can be seen that there are four living embryos alive at day 5 for trial 1. Two of these embryos were controls, and two of these embryos were experimentals. By day 5 for trial 2, only one control was still living, and there were two living experimental chicks at day 5 for trial 3.

However, not only did the embryos have to live for at least three days to be considered for quantification, but they also had to produce clear images of their hearts in order to be measured. Using the second qualification, only two of the seven embryos, one control and one experimental, which lived to day 5, could be used to collect quantifiable data. The control was from trial 2, and the experimental was from trial 3. These two embryos were used to compare the pulse and heart strength of chicks given daily doses of a 0.2 mg/mL caffeine solution.

Both the control embryo and the experimental embryo had much slower pulses than all of the embryos from the first trial, but they were still able to be compared because the embryos were kept in the same environment and exposed to the same variables, except caffeine, after explanting. Figure 2 below shows the change in the pulses over the course of three days.

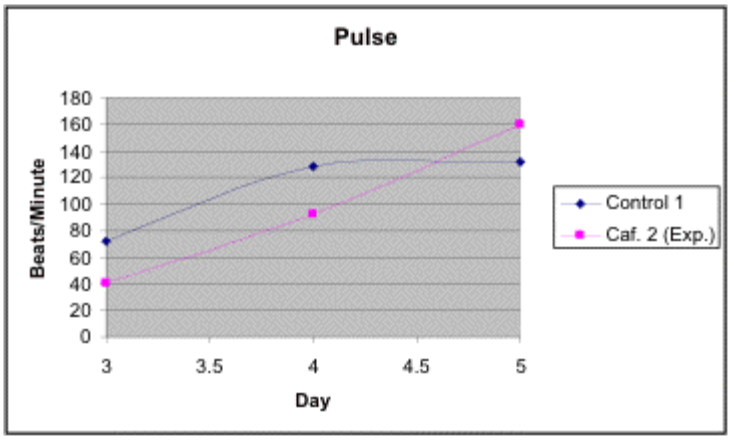


Figure 2: This graph shows the change in pulse over the course of three days for the control and experimental chicks. Refer to section 2.3 for the method used to determine the pulse.

The experimental chick, Caf. 2, had an extremely low pulse on the day that it was explanted, only 40 beats per minute. That is compared to 72 beats per minute for the control. Although the pulse started off abnormally slow, there were no other observable factors that differentiated the experimental chick from the control chick. By the third day after explanting, the experimental chick does have a higher pulse than the control because this is a steady increase in the pulse over those three days. The pulse for the control increases over the three days, but not as steadily or as much as the experimental embryo's pulse.

After graphing the heart strengths that were calculated with the formula given in section 2.4, a visible trend can be seen in Figure 3 below.

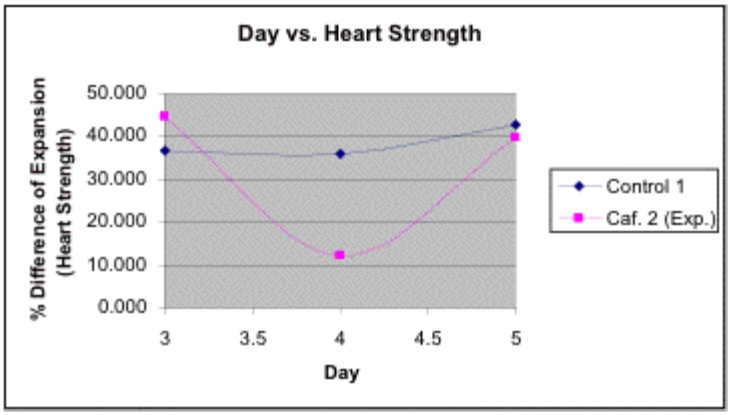


Figure 3: This figure shows the trends in heart strengths for the two embryos over the course of three days.

The heart strength for the experimental embryo on day 4 is obviously not consistent with the rest of the points, and the reason for this will be explained in the discussion. However, if only the general trend for the experimental is looked at, then it can be seen that there is a slight decrease in heart strength from the day the embryo was explanted to day 5. The trend for heart strength of the control embryo is slightly increasing over the three day period. It was hard to visibly observe changes in heart strength because of the growth that would take place each day, but the quantification shows

the trends.

4. Discussion:

There were two hypotheses made before the experiment was conducted, and both were supported by the results. The first was that the pulse for the experimental chicks will increase relative to the control chicks. The second was that the heart strength for the chicks given the daily dose of caffeine solution will decrease compared to the control chicks because having a prolonged, quickened pulse will cause the heart to be weaker. Figure 2 and Figure 3 in the results section show the steady increase in pulse and the slight decline in heart strength for the experimental embryos respectively, which is needed to provide evidentiary support for the hypotheses.

Since the hypotheses were supported by the data that was collected and analyzed, it can be concluded that chronic caffeine exposure to developing chicks decreases heart strength. Because the heart is responsible for pumping blood that carries oxygen throughout the embryo, a decrease in heart strength can lead to a decrease in blood flow and oxygen transport. Other studies have shown that the amount of oxygen delivered to a developing embryo is highly controlled, so any change in the amount of oxygen may lead to abnormalities of limbs, the heart, and the central nervous system (Webster W.S. & Abela, D., 2007). If a decrease in heart strength does change the amount of oxygen the embryo is receiving, which can lead to birth defects, then women should curtail their caffeine intake during pregnancy to reduce the chance of any of these effects.

Although the results support the hypotheses, there were some sources of error while performing this experiment and taking measurements that should be addressed. One source of error is for the second trial, which the data from the control embryo came from. All of the chicks from the second trial were held at 18°C for nine days, when they are usually only held at that temperature for two or three. This definitely affected the survivability of the embryos because only three were successfully explanted out of eight embryos, and only one lived past the first day. However, the control that was used to collect and analyze data did not have anything visibly wrong with it, but the pulse did seem slow the first day.

Some other errors that may have occurred happened while gathering data and taking measurements. My lab partner, Liz McKay, and I would alternate giving the chicks their daily dose of either Tyrode's solution or the caffeine solution. Caffeine is absorbed quickly by the embryo, so if the pulse and heart strength measurements weren't taken immediately after the solutions were given, then the data may not be an accurate reflection of the actual pulse and heart strength. Taking videos of the embryos' hearts proved to be a larger challenge than originally thought because there was not always a clear view of the heart due to the position of the chick. This eliminated five embryos from being used

for data analysis because the data couldn't be collected.

Even with the control and the experimental chicks used, the visibility of the heart proved to cause some problems. Only a portion of the heart was visible on day 5 for the control embryo. The largest possible line was measured across the visible portion of the heart, but that may not be completely accurate. For day 4 of the experimental chick, which was mentioned earlier, the chick was in a different position, which caused the angle of the heart to be different. Therefore, the measurement for that day was not consistent with the measurements from the day before or the day after. However, these two chicks gave the best views of the hearts for the other days, which is why they were chosen.

To make this experiment better for it to be performed in the future, there are a few things that should be changed. The sources of error above are the first things that should be addressed. To eliminate the problems, a larger sampling of chicks needs to be used. If the number of explanted chicks was in the hundreds, then after eliminating chicks for data analysis on the basis of the two qualifications listed in the results section, there should still be multiple control and experimental chicks to collect and analyze data from. With more than one control and experimental embryo, averages of the data can be taken to make the results more scientific, rather than anecdotal. Also, only one person should give the embryos their daily doses of solutions at the same time every day to make the results as accurate as possible. One other variable that should be kept constant if the experiment is repeated is the incubation period at 18°C.

More experiments should be done to solidify the conclusion that pregnant women should curtail their caffeine consumption because decreased heart strength in the fetus can reduce blood flow and oxygen transport, which may lead to birth defects. More research should be done on fetal hypoxia, decrease in oxygen circulation, during development. A suitable deviation that the oxygen level can fluctuate within so birth defects do not ensue should be found. The experiment should also be repeated with different caffeine concentrations. Concentrations both lower and higher than 0.2 mg/mL should be used in hope of finding a threshold where heart strength is not decreased. This could give pregnant women a limit for how much caffeine is safe to consume.

5. References:

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McKay, Elizabeth. Lab partner-helped explant chicks and administer caffeine and Tyrode’s solution throughout the experiment.

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