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RESEARCH ARTICLE

Chronic Hypoxia Induces Cardiomegaly and Increased Blood Flow Velocity during Chicken Heart Development

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ABSTRACT: This research examines the effects of hypoxia—low oxygen conditions—on embryonic heart development, specifically on heart size and blood flow velocities as markers of further defects. These early markers can be associated with later defects that come from these conditions, and explore the implications of hypoxia in all species' development. Fertilized chicken eggs (*Gallus gallus domesticus*) wrapped in clay and aluminum foil were used to model hypoxic embryo conditions. Each egg was incubated until approximately HH31 (day 7) or approximately HH35 (day 9). Then each egg was windowed, imaged using an ultrasound (Vevo 2100), dissected, weighed, and imaged under a microscope. We used the Doppler ultrasound feature of the Vevo 2100 system to determine flow velocity along the heart outflow tract. ImageJ was used to find the lengths and widths of the hearts from microscopic images by calibrating the size of the heart to the size of a known object, in our case, a 0.385 mm wide wire. Our results show that embryos that developed in hypoxic conditions had both larger hearts and faster cardiac blood flow velocities than control embryos, demonstrating that development in hypoxic conditions leads to abnormal development—an enlarged heart and faster blood flow— that perhaps can be projected onto humans.

KEYWORDS:Biomedical Engineering, Cardiovascular System, Hypoxia, Chicken Embryos, Blood Flow Velocity.

Introduction

Problem Statement and Research Aim:

In cases of chronic hypoxia during pregnancy, the developing cardiovascular system may adapt by developing heart defects. The developmental plasticity of the embryo allows it to compensate for the decreased oxygen levels, but this adaptation can lead to ventricular septal defects (VSD), atrial septal defects (ASD), or patent ductus arteriosus (PDA), which are heart malformations at birth. In this study, we focused on the effects of chronic hypoxia.

Congenital heart defects (CHD) are present in about 1% of births. VSDs are the most common CHD and account for 37% to 64% of cases of CHD or about 0.3% of births.[1] Moreover, VSDs have been studied as a cause of chronic hypoxia rather than a side effect. Except in cases of pulmonary arterial hypertension (PAH), the mortality rate for VSDs post-operation is about 1%.[2] Blood flow (hemodynamics) during pregnancy is key in understanding the development of CHDs, such as VSD, as well as the redirection of blood flow due to malformations. During pregnancy, the placenta forms as a temporary organ and the source of nutrients, oxygen, and waste filtration for the developing fetus. The fetus is attached to the placenta by the umbilical cord and depends on the mother to act as a sole source of resources.[3] Because of the position within the uterus, the fetus is unable to breathe in oxygen with its own circulation system. The dependence on the mother for oxygen can lead to a variety of complications in the case of placental, umbilical, and maternal conditions that may cause chronic hypoxia. Compression of the umbilical cord restricts oxygen to the fetus, as does any issue concerning the placenta, such as placental infarction, altered development, or poor placental function. In cases of maternal diseases such as preeclampsia, hemoglobinopathy, pulmonary hypertension, anemia, substance abuse, high altitude during gestation, and low blood pressure, chronic hypoxia is inflicted upon the fetus.[4] During human pregnancy, cardiomyocytes (the muscle cells of the heart) begin to contract 16 days into development.[5] Since cardiomyocytes continue to contract during development, pumping blood to the embryo, embryogenesis depends on the circulation system. In cases of chronic hypoxia, partially oxygenated blood is circulated, affecting developing tissues and forcing the fetus to compensate by redirecting blood flow and even developing cardiomyocyte hypertrophy.[6-9] These developmental changes may affect the individual later in life, through cases of congenital heart disease.

Current research on hypoxia (using chickens as a model organism) has focused heavily on the rate of development, long-term outcomes, cardiac mass and proportions, epigenetics, and resulting heart defects.[10-13] More recently, doctoral student Nina Kraus from the University of Vienna, Austria, established a procedure using clay to emulate chronic hypoxia in chicken embryos. Because chicken embryos breathe through their egg shell, the clay creates hypoxic conditions. The goal of our research is to identify how chronic hypoxia affects cardiac development, blood flow, and cardiac anomalies in chicken embryos. The focus on hemodynamics, as opposed to long-term outcomes and heart mass, has been stressed in this research.

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Chicken Embryos as Model Organisms:

Avian embryos are frequently used as models of cardiac development because they have a relatively quick incubation period of 21 days, and their hearts develop in a similar pattern to the human heart, resulting in a four-chamber configuration. Developmental programs, moreover, are highly conserved in vertebrate species. Chicken embryos can thus be used to study the effects of chronic hypoxia on hemodynamics and heart development. Hamburger-Hamilton (HH) Stages identify the progression of chicken development during incubation. The method of staging comes from a study done by Hamburger and Hamilton in 1951. Images of the stages are shown in Figure 1.

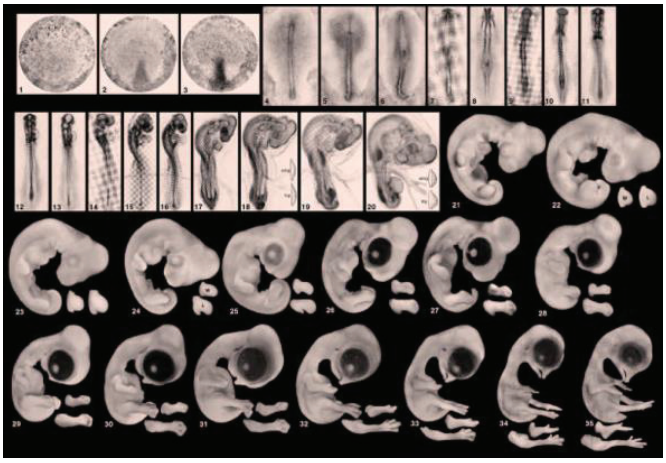

Figure 1: Hamburger Hamilton stages of chick embryo development. Used to accurately remeasure stages of growth throughout the experiment.[14]

HH stages identify how developing embryos change over time (during the 3 weeks of incubation) and the distinct characteristics they develop. There are 46 total stages of chick incubation, with HH46 being the final one and representing a hatched chick. At stage HH10 of embryo development, cardiac cells of a primitive tubular heart begin to contract, establishing embryonic circulation.[15] At HH31 (approximately day 7), the outflow tract continues to septate into vessels to provide oxygen and nutrients to the developing organs. The coronary vessels are evenly spaced, and the aortic and pulmonary valve leaflets have changed position to angle in towards each other. At HH35 (approximately day 9), the outflow tract and ventricles have fully septated, and the semilunar valves have finished developing. Cardiac neural crest cells spread through the cardiac plexus (the network of nerves located at the base of the heart) to prepare for the development of the peripheral conduction system (the Purkinje fibers). During embryological development, the chicken embryo receives oxygen through a system of gas exchange. When the egg is laid, the inner membrane shrinks slightly, creating an air pocket at the blunt end of the egg. This area grows larger as the egg ages, due to moisture diffusing out of the shell, and needs to remain uncovered for proper growth. Gas diffusion occurs through the pores on the shell of the egg, and therefore, claying (or covering) around half of these pores would create a hypoxic environment, without completely cutting off oxygen.[16] In our research, we used chicken embryos as our model organisms to study the effect of chronic hypoxia on cardiac development. To do so, we used clay wrapped around the bottom portion of the egg (blunt side up). Gas exchange occurs through the shell into the area between the inner and outer membranes, so the clay restricts access to oxygen (without completely blocking it), simulating chronic hypoxic conditions characterized by a reduction in oxygen for an extended period of development.

Methods

Fertilized chicken eggs were first clayed at day 2 or 3 of incubation to simulate hypoxic conditions. They were then incubated until day 7 or day 9, when they were imaged through an ultrasound to measure blood velocity through the heart. Once imaged, the embryos were dissected to remove the hearts, which were then either frozen in optimal cutting temperature compound (O.C.T. gel) in preparation for histological slicing to determine structure under a microscope or preserved in phosphate-buffered saline (PBS). The hearts that were preserved in PBS were then weighed. The frozen hearts were cut via cryostat in 10 μm thick slices, then stained using either a hematoxylin and eosin (H&E) stain or a Polysciences Differential Quik Stain. Under the microscope, they were measured in comparison to a 0.385 mm craft wire to find the length and width of each embryo's heart. We performed experiments in three sequential batches, or trials, adjusting techniques as we were learning to perform the studies and as needed. All eggs were incubated in an approximately 37°C incubator with 65%-80% humidity.

Claying:

Trial 1 included 26 eggs, which were split into 13 controls and 13 experimental clay-covered eggs. To begin trial 1, the bottom half of the experimental clay-covered eggs was wrapped with clay on day 3, then aluminum foil (to prevent flaking), leaving the blunt end uncovered. This created a hypoxic environment by eliminating oxygen diffusion through half of the shell. At day 3 of incubation, the embryos were at approximately HH18, where the heart, which is tubular at this stage, is in an S-shaped loop. The 13 control eggs were not covered with clay. All 26 eggs were windowed on day 3 using curved and straight forceps, and the outer embryo membrane was removed using forceps to make the embryos visible. The windows were then covered with plastic wrap and secured with glue, see Figure 2 (left). Trial one included 2 experimental groups: control and day 3 clayed embryos (clayed D3).

Trial 2 included 21 eggs, which were split into 5 controls and 16 experimental clay-covered eggs. Of the 16 clay-covered eggs, 8 were covered with clay on day 2 of development, and 8 were covered with clay on day 3 of development (Figure 2). At day 2, eggs were at approximately HH stages 12-13, where dextral looping of the heart begins. At these stages, the endocardial cushions also emerge (precursors to valves). The trial 2 eggs were kept unwindowed until day 7 of incubation, see Figure 2 (right). At day 7, the eggs were at stage HH31-32. By HH31, the distal portion of the outflow tract has finished septating, the coronary arteries and veins are in their final positions, and the aortic and pulmonary valves are angled.[15] Trial 2

included 3 experimental groups: control, day 2 clayed embryos, and day 3 clayed embryos.

Trial 3 included 15 eggs, which were split into 6 controls and 9 experimental clay-covered eggs. All 9 experimental eggs were covered with clay on day 3 of development. The trial 3 eggs were kept unwindowed until day 9 of development. On day 9 of development, the eggs were around stage HH35-36, at which the Phalanges in the toes developed.

Figure 2: Trial 1 eggs (left) are shown windowed and clayed. Trial 2 eggs (right) are shown only clayed. Used as a model for what a recreatable setup would include.

Experimental groups:

In 7 groups were used in this experiment: i) control incubated until day 7 (windowed), ii) clayed day 3 and incubated until day 7 (windowed), iii) control incubated until day 7, iv) clayed day 2 and incubated until day 7, v) clayed day 3 and incubated until day 7, vi) control incubated until day 9 and vii) clayed day 3 and incubated until day 9. Trial 1 included control incubated until day 7 (windowed) and clayed day 3 and incubated until day 7 (windowed). Trial 2 included control incubated until day 7, clayed day 2, and incubated until day 7, and clayed day 3 and incubated until day 7. Trial 3 included a control incubated until day 9 and clayed on day 3 and incubated until day 9.

Ultrasound:

On day 7 or 9, eggs were taken out of the incubator to perform in vivo ultrasound imaging via a FujiFilm VisualSonics Vevo 2100 Imaging System. Trial 1 eggs, which were previously windowed, were windowed further to increase space for the ultrasound transducer. In trials 2 and 3, eggs were windowed for the first time. The windowed egg was then placed into a special 3D printed egg holder atop a rising platform, which had the transducer mounted above. The egg was then raised so that the embryo could touch the transducer. Using the visual displayed on the ultrasound screen (Figure 3), the egg was positioned by hand to get the right angle. To measure the pulse wave velocity, the heart outflow tract, which directs blood from the heart to the circulation, had to be clearly visible, and the color Doppler feature of the Vevo system helped find it (Figure 4). Once the outflow tract was identified, the ultrasound's Vevo Lab software was used to measure blood flow velocity through the outflow tract. Peak and mean velocities (mm/s) were taken from three pulses (Figure 5). This process was repeated for each egg. Some eggs could not be imaged properly due to their position or death during the process.

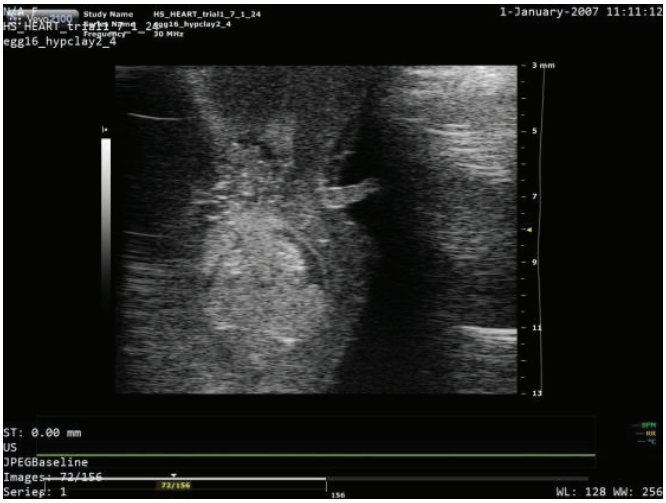

Figure 3: Normal B-mode scan with a 4-chamber view of the heart. Shows a regular-sized heart and is used as a model to ensure that all scans are imaged at similar angles to get reproducible results.

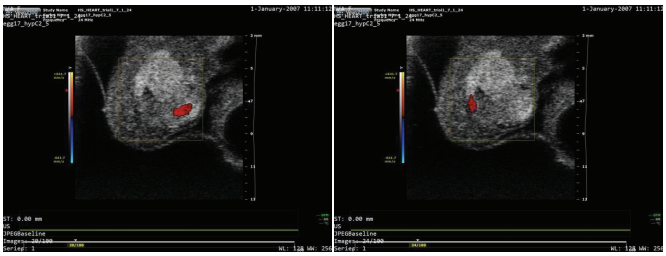

Figure 4: Colored Doppler images. Red indicates blood flow toward the transducer. Blue indicates blood flow away from the transducer. Shows imaging angle for reproducible results. Shows which heart valves were measured to achieve velocity measurements.

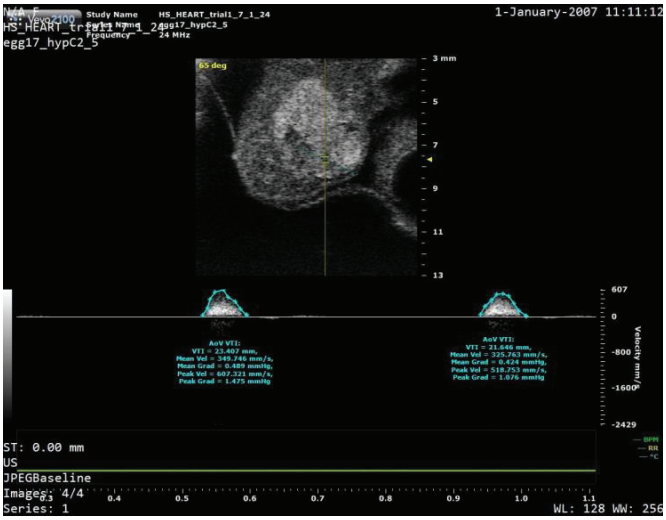

Figure 5: Pulse wave doppler mode, velocity measurement. Shows how average and peak velocities were obtained for reproducibility. Shows the heart of a hypoxic embryo with relatively high blood flow velocity.

Dissection:

Once an embryo's in vivo ultrasound imaging was complete, the embryo was removed from its shell for dissection. The embryo was transferred from its shell to a petri dish (Figure 6) using a scoopula and then decapitated using a scalpel. The ribcage was then cut open using microdissection scissors and peeled away using forceps, exposing the heart (Figure 7).

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Scissors were used to cut around the heart, removing as much excess tissue as possible. Once completely separate, a transfer pipette was used to place 1-3 drops of potassium chloride (KCl) on the heart to ensure it would stop beating and the muscle would fully relax. Finally, the heart was placed in a Tissue-Tek cryomold and covered with O.C.T. gel before freezing. Samples were frozen via dry ice and then placed in a -80°C freezer.

Figure 6: Chicken embryo removed from egg before dissection. The embryo is on day 9 of incubation. Shows how chicken embryos were analyzed for abnormalities that Nina Kraus found.

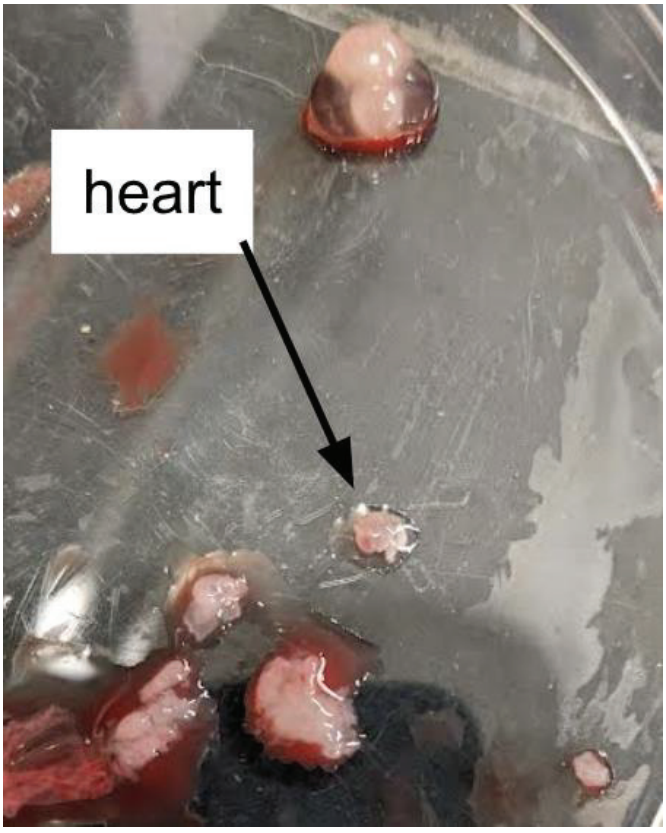

Figure 7: Labelled heart post-dissection. The heart is from day 9 of incubation. Shows how a hypoxic heart was dissected for reproducibility.

Cryostat:

Once frozen, a Leica CM1860 cryostat (Figure 8) was used to cut the O.C.T. block with the frozen hearts embedded in it into 10 µm thick slices for histology slide preparation. First, the cryostat's slice thickness was set to 50 µm. The heart, covered in a frozen square of O.C.T. gel, was taken out of its cryomold and placed into the cryostat's mount. The wheel on the side of the cryostat was cranked to move the mount down into the blade, to cut away excess O.C.T. gel. Once the desired amount was removed, the glass anti-roll shield and blade were fixed in place, and the slice thickness was set to 10 µm. Once one slice was cut, it was placed on a glass slide, and the O.C.T. was allowed to melt while the tissue stuck to the glass. The process was repeated until the whole heart was sectioned.

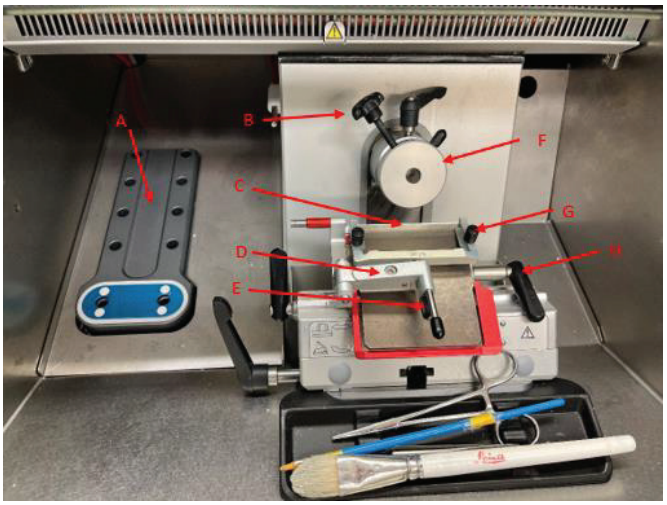

Figure 8: View of Leica CM1860 cryostat work area. (A) Tissue storage area. (B) Tightening knob. (C) Microtome blade. (D) Glass anti-roll plate holder. (E) Anti-roll plate adjustment knob. (F) Chuck. (G) Glass plate tightening screws. (H) Microtome blade clamp lever. Used for reproducibility.

Staining:

There were two different methods of staining that we used, and heart sections were stained with one or the other (it is not possible to stain with both simultaneously). The first was a basic hematoxylin and eosin (H&E) histological stain. The H&E staining process included placing the heart slides in baths of different chemicals and letting them sit for different time intervals. This included two baths of phosphate-buffered saline (10 minutes each), one of hematoxylin (3 minutes), a rinse of deionized water, then one bath of eosin (30 seconds), one of 50% ethanol (1 minute), one of 90% ethanol (1 minute), two of 100% ethanol (1 minute each), and two of xylene (10 minutes each). Once complete, the stain highlighted cell nuclei, cytoplasm, and extracellular matrix. Slides were then covered with cover slips and were ready for imaging.

The second staining method used the Polysciences Differential Quik Stain, which had only three steps. The heart slides were first dipped into the fixative (methanol), then Solution A (an eosin-based stain), then Solution B (methylene blue). The slides were then covered with cover slips and ready for imaging. This stain highlighted nearly all cells.

Microscope:

The microscope used in this experiment was a Leica stereo microscope M125 C, along with the pco.camware that recorded movies or still images of the hearts. ImageJ (an open-source, free imaging software) was used to measure the length and width of each embryo's heart from the cryo-sections. With the slide positioned under the microscope, a piece of 0.385 mm silver-plated craft wire was placed on the slide next to a chosen heart. The camera was zoomed in to view the chosen heart and wire, and the focus was adjusted to view both objects clearly. An image was captured and opened in ImageJ, where lines were drawn across the wire diameter, length of heart, and width of heart (Figure 9). Lines were measured (in pixels) of each of the lines, and a ratio was created of the wire diameter in pixels and millimeters. The ratio was then used to calculate the heart width and length in millimeters.

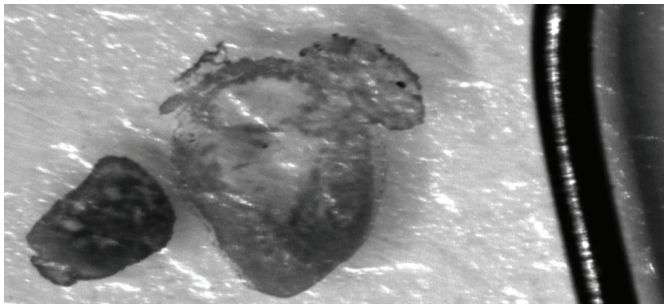

Figure 9: Image of day 7 embryo heart from pco.camware software. 0.385 mm wire located on the right side. Shows a relatively large hypoxic heart and the process for analysis for reproducibility.

Weighing:

6 hearts incubated until day 7(1 control, 3 clayed day 2, 2 clayed day 3) and 4 hearts incubated until day 9 (1 control, 3 clayed day 3) were preserved in tubes with PBS. To weigh, a Sartorius Precision Scale was zeroed to the weight of one weigh boat. Then, a heart was removed from its tube using straight forceps and placed onto the weigh boat on the scale. Mass was recorded in grams. The process was repeated for each heart.

Result and Discussion

Because eggs were windowed early (day 3) for trial one, and we suspect the window enabled additional oxygenation through the window, we only used the results of trials 2 and 3 for analysis. Instead, we used trial 1 embryos for practice and to test our techniques.

Our data show that chicks inside eggs wrapped in clay to simulate hypoxia had (at day 7) a faster blood flow velocity through the outflow tract than control embryos, both for embryos inside eggs clayed at day 2 or day 3. Eggs were divided into 3 experimental groups: control, clayed on day 2, and clayed on day 3. On day 7 of incubation, the average mean blood flow velocity increased by 62% from the control embryos for embryos clayed on day 2 of incubation and increased by 66% from the control embryos for embryos clayed on day 3 of incubation. Figures 10 and 11 show graphs of average mean velocities and average peak velocities to display this behavior. These results show that hypoxic embryos had, on average, developed both a faster mean blood flow and a higher peak blood flow when compared to the control embryos. But there was no significant difference between eggs clayed on day 2 and eggs clayed on day 3. Figure 10 has outliers, which were better addressed in Figure 11 by averaging out the results.

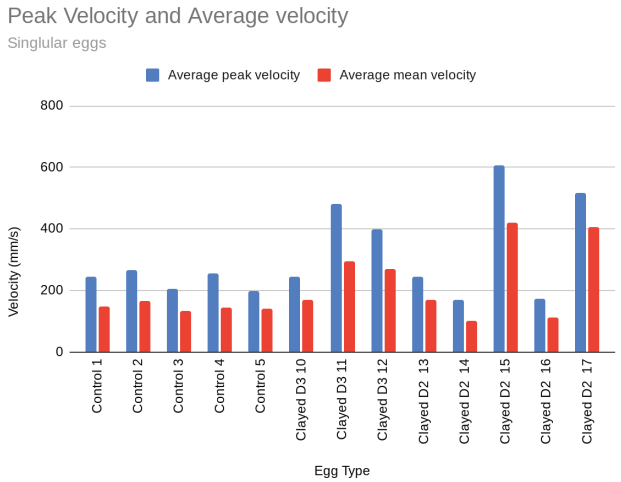

Figure 10: Individual measurements of peak and mean blood flow velocities through the ventricular outflow tract at day 7. Higher peak and average blood flow velocities can be seen among the embryos that were clayed.

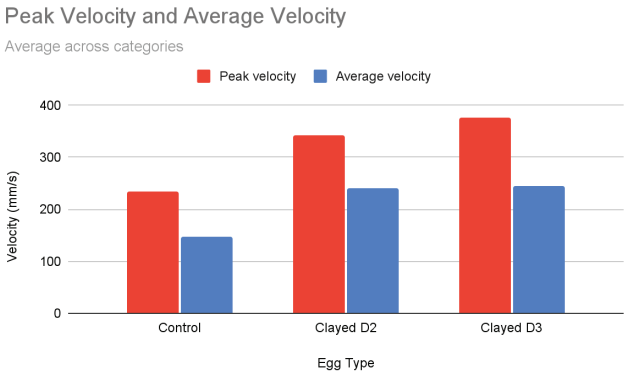

Figure 11: Measured blood flow velocities of the outflow tract of day 7 chick embryos. When averaged across categories, there is a significant increase in velocity among clayed eggs.

We also measured the same data for eggs that incubated until day 9 of development and found similar results. In Figures 12 and 13, we can see that both the mean and peak velocities stayed higher than the control embryos. This data shows that the higher blood velocity did not go away with development. Higher mean and peak velocities persisted over time for hypoxic embryos.

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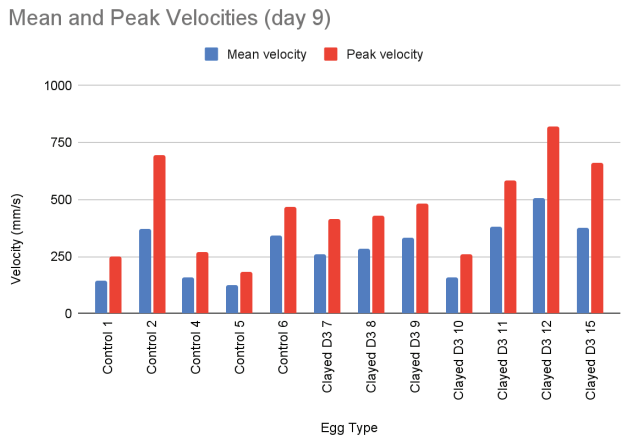

Figure 12: Individual measurements of blood velocities through ventricular outflow (day 9). Among the eggs incubated until day 9, there is an increased blood flow velocity among the clayed eggs, with some outliers in the control group.

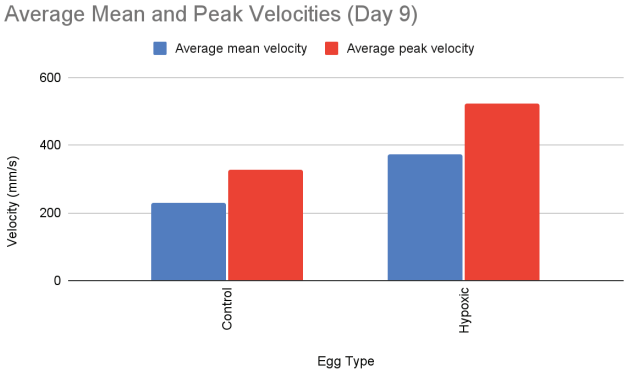

Figure 13: Average blood flow velocities of the outflow tract of day 9 chick embryos. When averaged across groups, there is a significant increase in the blood flow velocities among the clayed/hypoxic eggs.

Additionally, we measured the length and width of the clayed and control embryos' hearts. From stained sections of the hearts, approximately corresponding to the middle of the heart, where the heart is larger and wider, we found that, on average, the hypoxic embryos developed both longer and wider hearts. Figures 14 and 15 show that the hypoxic (clayed) embryo hearts were larger than the control embryo's hearts. While cutting the hearts to put onto the microscope slides, some of the hearts were sectioned in an incorrect orientation, and therefore, we could not get both length and width, but only one or the other. That is why some heart measurements only have length or width measurements. Also, some embryos were not processed for staining, hence we have data from fewer embryos than for previous measurements. These results show that the hypoxic embryos' hearts developed to a larger size. There was not enough data to determine differences between eggs that were clayed on day 2 and eggs that were clayed on day 3 of development.

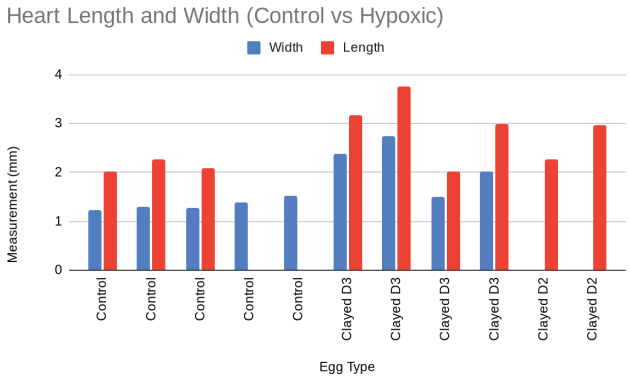

Figure 14: Individual measurements for heart sizes for each group of eggs (day 7). It can be seen that there was a slight increase in the heart size among the eggs that were clayed compared to the control group.

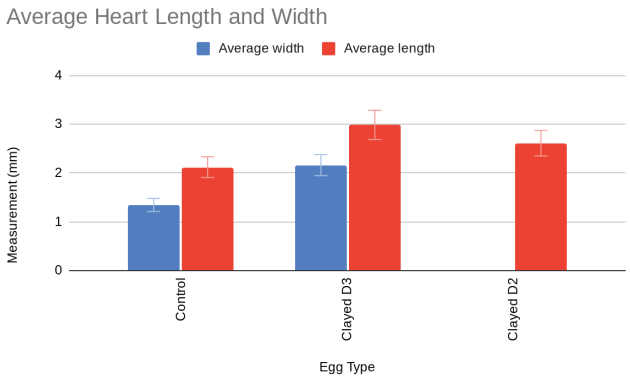

Figure 15: Average heart size for each group of embryos (day 7). When averaged across groups, a slight increase can be seen in the heart size of the control/hypoxic eggs when compared to the control groups.

Our results also show that the hypoxic embryos developed slightly heavier hearts. Not all the hearts that were dissected were weighed, so the data set is smaller than the other experiments, and only includes 1 control heart, which is not ideal. Figures 16 and 17 show that, on average, the hearts that developed in hypoxic conditions weighed more than those that developed normally. This shows the larger heart size was due to an actual increase of cardiac mass in the hypoxic embryos. These findings are significantly hindered by a limited sample size.

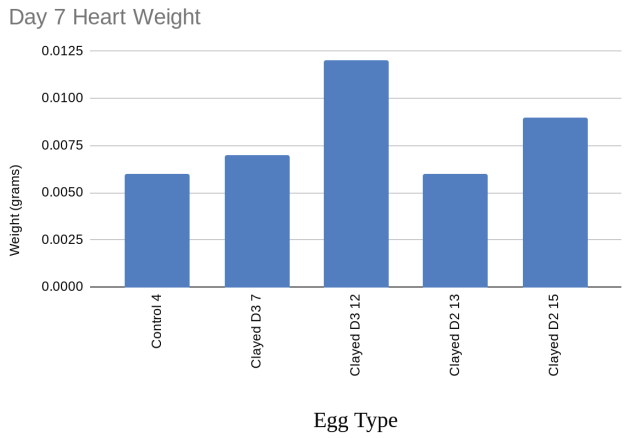

Figure 16: Individual measurements of the weight of the heart for each group (dissected on day 7). This figure shows a slight increase in the cardiac mass among the clayed embryos when compared to the control groups. This figure supports the previous findings and implies that the increase in cardiac size is due to increased cardiac mass.

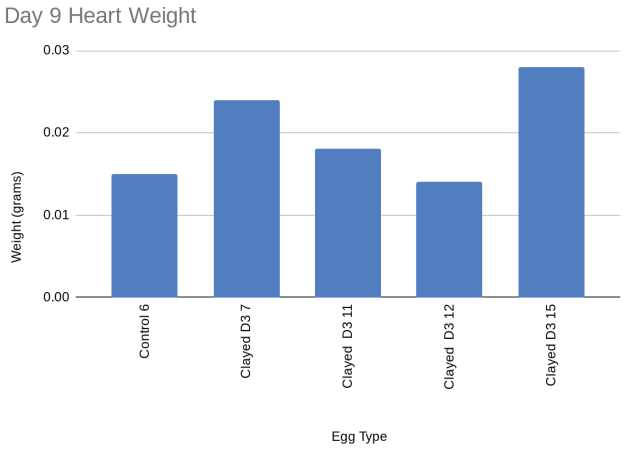

Figure 17: Individual weights of hearts for each group (dissected day 9). Similar to the previous figure, this shows a slight increase in cardiac mass among the clayed eggs and implies that the increase in heart size is due to increased cardiac mass.

The first trial of windowed eggs could not be used for data, as the method for windowing the eggs exposed the embryos to excess air, which made the trial unsuitable to induce hypoxia or to provide a suitable control reference. The sample size in this experiment was limited due to several issues, such as i) one of the incubators that was used did not maintain ideal humidity due to frequent use (opening of the door) and decreased viability, ii) we could not measure the ratios of heart size to embryo size due to no measurements of embryos. This research was also done on a limited 3-week period, which did not allow for as much testing as we wanted, and limited time to incubate eggs. In further research, the same experiment could be conducted with a larger sample size (15-30 viable chicken embryos per experimental group), and by leaving the eggs to incubate longer (until at least day 12/13), observations of further heart defects could be conducted.

Using Welch's t-test to test for statistical significance, it was found that when comparing control vs clayed egg blood flow velocities, both peak and average velocities attained a value of $p<0.01$ ($p=0.0022$ and $p=0.001$, respectively, along with t values of -3.39 and -3.79). This signifies that the results are significant, and both measures of blood flow velocities were significantly higher in clayed groups. For weight, there were not enough samples to run a statistical significance test, so more samples would need to be taken.

Conclusion

Mothers with placental issues or locational issues that can cause hypoxia are more likely to deliver babies with congenital heart defects. Around 1% of babies in the US are born with congenital heart defects, and 1 in 4 of these are critical heart defects.[16] These congenital heart defects form in the early stages of embryonic development and are very susceptible to different variables, especially a mother's placental health and choices, which can affect both changes in the embryo's access to oxygen and blood flow in the embryo's heart. Both reduced oxygen and altered blood flow in hypoxic embryos can lead to heart defects. Previous research has not investigated variability in heart sizes and weights in early stages of life due to hypoxic conditions, nor changes in blood flow velocities in the heart. To our surprise, we found that hypoxic hearts are larger than control hearts (cardiomegaly), and blood flow velocities in hypoxic embryos are increased with respect to the control. We did not notice other malformations with reference to control hearts.

In our research, we used chicken embryos to model heart development and study the effect of hypoxia on heart development. Based on our experimental results, we found that embryos that develop in hypoxic conditions have altered heart development, but more measurements will need to be done in the future to confirm our results. Along with this, this experiment is limited due to the use of wet measurements for heart weight; future experiments could include dry measurements to determine further if heart growth was due to edema or tissue growth. This experiment could also be expanded to other animal models—quail, zebrafish, mice—to observe if similar results occur. More measurements could be taken to study other complications. Running this experiment several times could eliminate some errors, increase our certainty, and provide better insight into hypoxic heart development.

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