Ethanol-exposed chick embryos exhibit lower vascular areas, increased heart rates and longer body lengths.

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I. Introduction

Since Aristotle chick embryos have been used to study development for several reasons. Firstly, they are easily obtained in all seasons (Daley, 2002). Secondly, they also teach us a great deal about the structures that are the diagnostic characters of the phylum Chordata and subphylum Verterbrata, making it possible to extrapolate from chick to human and know about what must go right for a normal baby to be born without defects (Tyler et al., 2004). In this investigation, we will use explant culture to study the effects of ethanol (EtOH) exposure on the development of a vascular system, particularly on cardiogenesis, in a 72-hour chick embryo. The cardiovascular system is unique among organ systems in that it must function in the early stages of its own development, or the rest of the embryo will fail to develop normally. This is because complex organisms outstrip their capacity to receive nutrients and oxygen by diffusion just as the development of their organ systems is beginning to take develop. An embryo requires properly routed oxygenated and deoxygenated blood to separate paths; an abnormally routed vessel or underdeveloped heart chambers quickly become a life-threatening problem (Fang et al., 1987).

72 hours of incubation is required to observe the effects on the ventricle, atrium and aortic arch, which have all developed by this time. Cardiogenesis is also underway by 72 hours (Ruckman, 1988). We will study the effect of ethanol as a teratogen (an agent which causes abnormal development) because maternal consumption of alcohol is believed to adversely affect the health of at least one in 750 infants born in the United States (Armstrong, 1994). Cardiogenesis is a critical sensitivity window, meaning that the cell is particularly susceptible to disturbances, or “developmental insults,” during this period in which cellular signals are directing the heart’s division into its various chambers and vessels. Retinoic acid (RA) is an essential regulator of morphogenesis during heart development; alcohol causes low levels of RA which result in apoptosis (programmed cell death) of developing cardiac populations (Chow, 1997). We will measure the degree to which alcohol perturbs and causes defects in a developing chick heart by measuring the heart rate. As the current data indicates, reduced embryonic heart rates during cardiogenesis are associated with the development of ethanol-induced intracardiac defects (Bruyere, 1994). We therefore hypothesize that when the chick embryo is exposed to ethanol the heart rate will slow down and palpitate as the blood cannot pump through the heart as easily; furthermore, visible defects, such as the shrinking of the artery should be present, as well as a lower vascular area. We will also measure vascular area, as we know from past studies that Retinol is crucial to differentiation of the vascular system and alcohol interferers with retinol regulation (Fang, 1987). Vitamin A, and its retinoic derivatives, are crucial morphogens in heart and vascular differentiation. In addition to heart rate and vascular area, body length will also be measured. We expect body length to be lower in ethanol-exposed embryos as past studies by Yang have indicated (Yang, 2004).

To test the hypothesis that vascular area, body length, and heart rate will decrease we will culture six chick embryos in explant culture. Explant culture is the process of transferring an embryo from an egg to a culture medium. We will culture the embryos in varying levels of alcohol and measure both the atrial and ventricular beats using a watch with a second hand while also measuring vascular area and body length using stereomicroscopy and digital imaging to examine the specimens for defects.

II. Materials and Methods

We began our experiment by cleaning the lab bench using paper towels and 70 % ethyl alcohol (EtOH). We also thoroughly disinfected our hands with the EtOH before handling the eggs. It is imperative that the area of work is sterile or semi-sterile to prevent infection, which could kill the embryos and throw off our data. We therefore disinfected each egg by wiping it gently with a paper towel moistened with a 70% EtOH solution.

The eggs were ordered from Charles River Laboratories and were held in stasis at low temperature until we took them for explanting and incubation. We explanted the embryos on day 3.

The explant began by cracking the wide end of the egg gently with sterilized forceps, and removing shell around the large end, exposing the air space. Once the edges of the shell were made smooth by forceps, another hole was made on the other side of each egg. As soon as air was let in the contents flowed out in most cases, however, one egg in the first batch displayed a broken yolk.

The above step was repeated for all the eggs that were explanted. To each dish 0.5 ml penstrep solution was added (penicillin and streptomycin) with a pipette. The penstrep was diluted by a 1:100 dilution factor. Alcohol was added to the eggs that were to be tested for the effects of ethanol exposure; other eggs were not treated with ethanol and were used as controls. Of the seven eggs that survived explant four were used as a control, two were given a higher ethanol concentration, and two were given a lower ethanol concentration.

To the solutions that received higher-concentration alcohol solutions, 2% EtOH was added to 0.5 mL penstrep solution to give a final EtOH concentration of approximately 0.02% (the volume of each egg is approximately 50 ml); in the two samples with lower concentration we added 0.2 % EtOH to 0.5 ml penstrep solution, giving a final EtOH concentration of 0.002%. Once the EtOH-penstrep solutions were added to the weighboats they were placed carefully in a 37 C incubator. Each was labeled control, 0.02%, or 0.002%, depending on their identity. Transport into the incubator was done carefully so as not to disturb the embryos or even tip them over. The corners of the weigh boats were folded up to keep lids from touching and contaminating the yolk, and to ensure air exchange.

The embryos were then observed daily beginning on the third day since incubation. After the three days ocular measurements were taken using a ruler with millimeters. For each part of the embryo being measured—vascular area, body length, wing bud length, etc.—the longest length across the feature was measured and then recorded in millimeters. The data was recorded first onto a table distributed in class and then onto Microsoft Excel, to make it easier to analyze and compare. Observations in the 72 hour period were not made as the embryo could not be measured ocularly. The whole class collected data on several parts of the chick embryo including the vascular area, body length, wing bud length, leg bud length, alantois width, and heart rate. Heart rate was measured simply, using a watch with a second hand during a period of one minute.

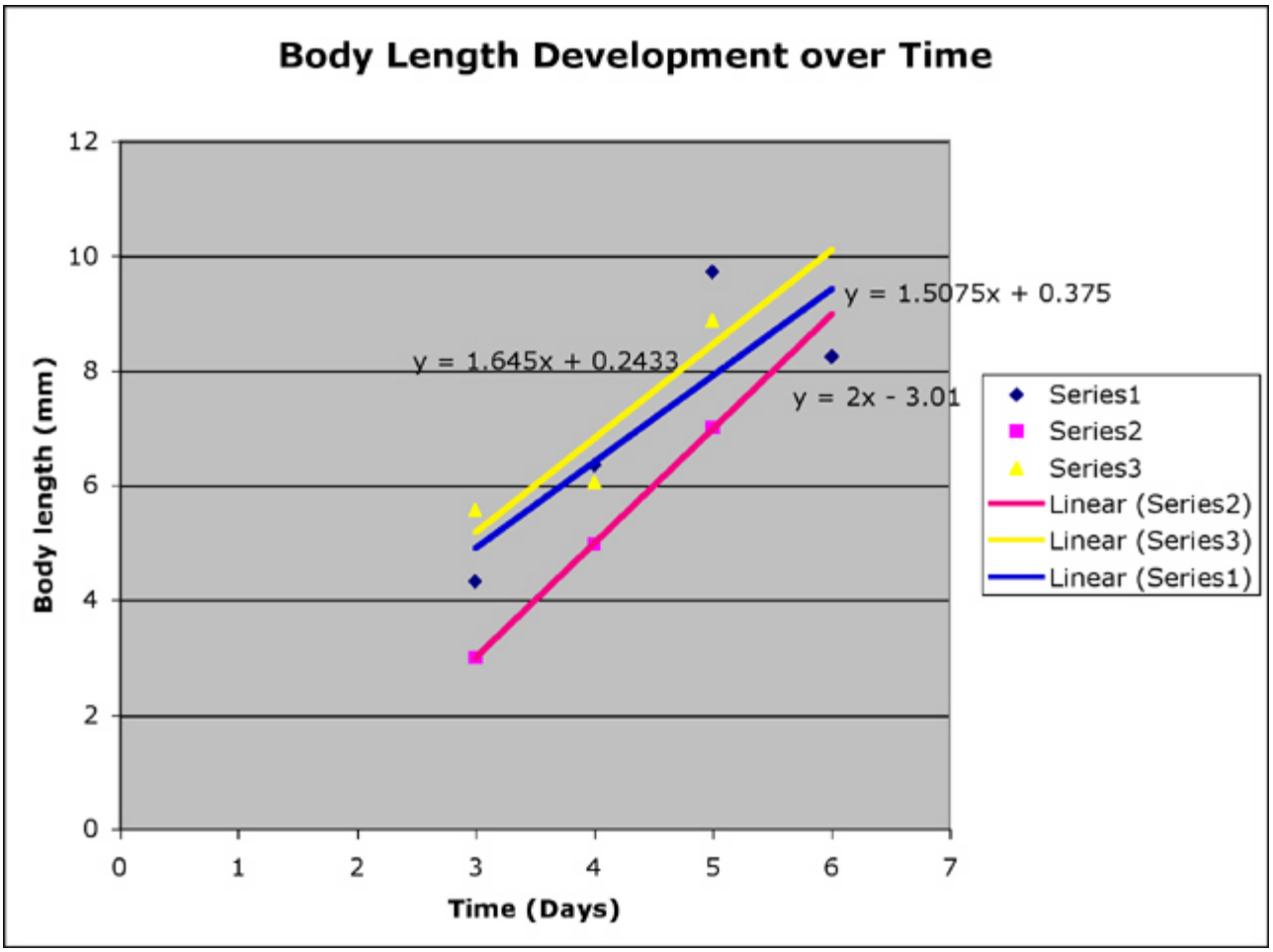
Averages of class data were collected, tabulated, analyzed and graphed using Microsoft Excel. When a chick died, it was not averaged but discluded entirely. Therefore some data series will have more values than others as more chicks died in certain concentrations. The data was pooled since there was no evidence that control data or experiment data should vary between trials or between groups executing the experiment.

The specimens were examined using stereo spectroscopy. A Nikon DAGE-MTI DC 200 dissecting microscope was used to examine the embryos for malformations. BTV Pro for Mac OS X was used to view the images being sent to the Mac G3 from the microscope.

III. Results

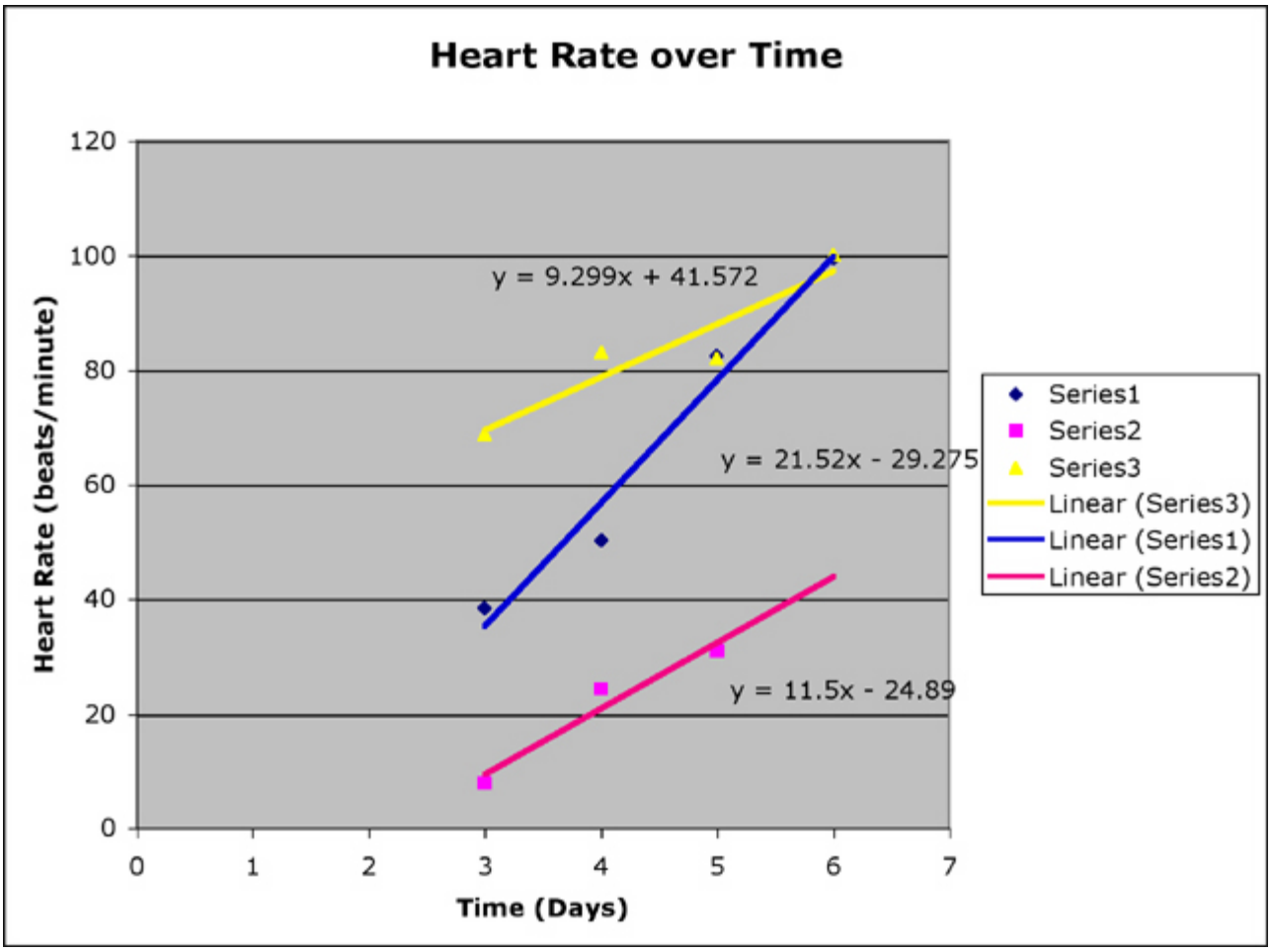
NOTE: In the following graphs, series 3 (yellow) refers to 0.02 % EtOH; series 1 (blue) refers to control; and series 3 (pink) refers to 0.002 % EtOH.

Figure 1: Body Length over Time



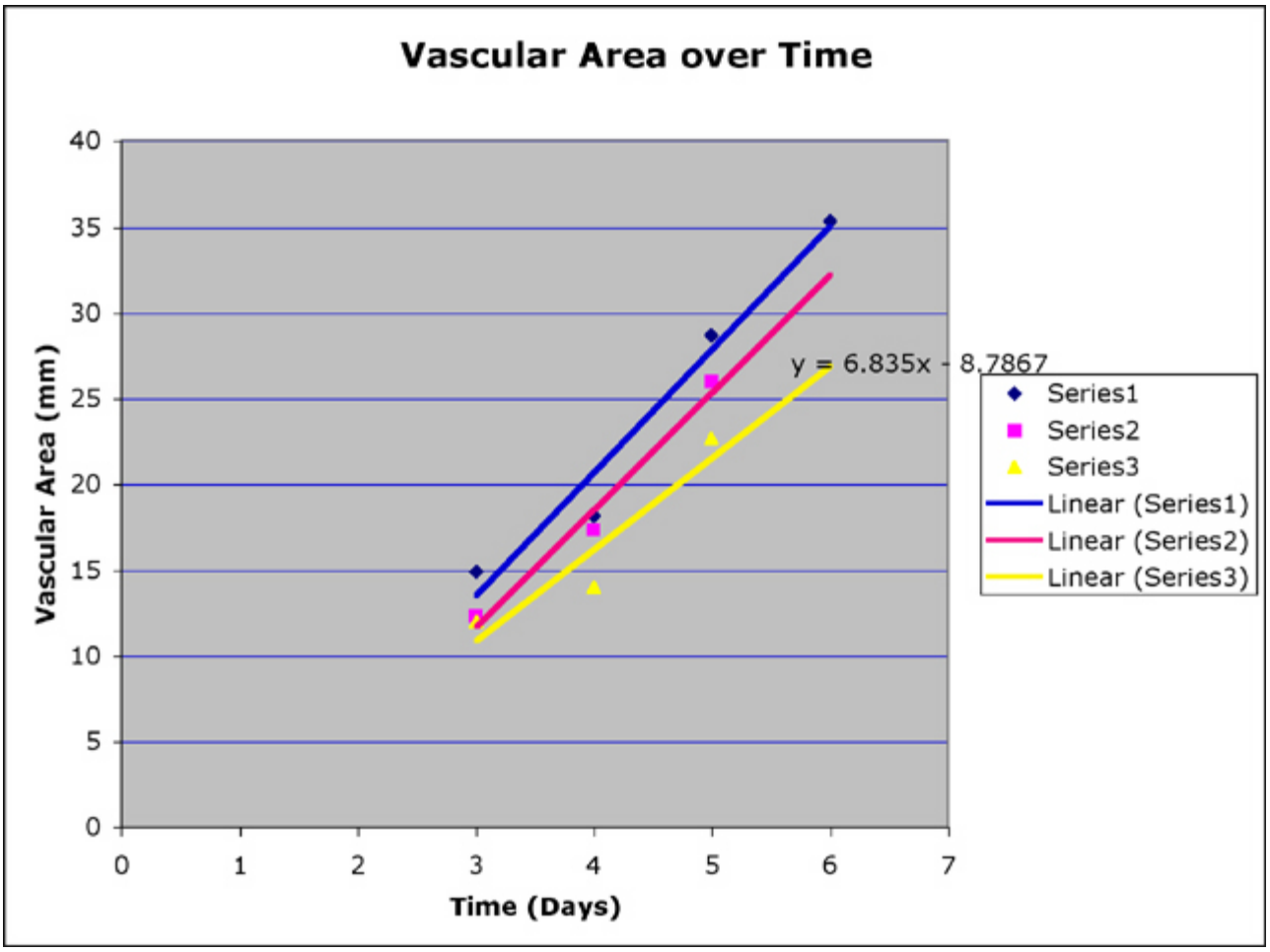
The above graph shows that high EtOH exposed embryos had a larger body length over time than the control embryos. The embryos exposed to low EtOH had the lowest body areas at each day observed but their body area is increasing at a faster rate than either of the other groups. The control group's body area is increasing at a slower rate than both the group exposed to higher ethanol and the group exposed to lower ethanol. This evidence contradicts our hypothesis. The body length of the low EtOH exposed group is growing at the fastest rate and will overtake both the body length of the control group and the body length of the high EtOH group if this trend were to continue.

Figure 2: Heart Rate over Time



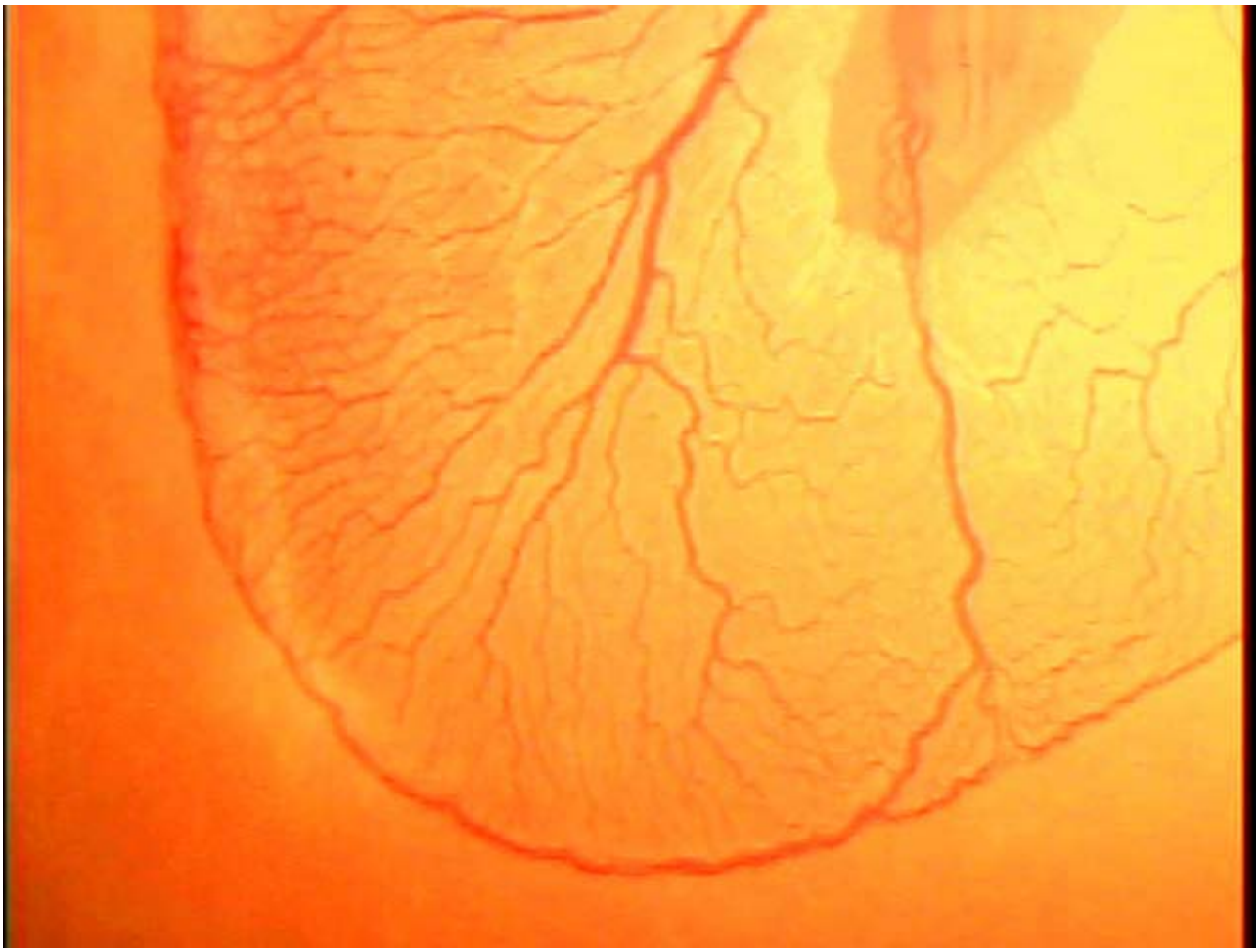
The above graph shows that although high EtOH exposed embryos started with the fastest heart rates, their heart rates did not increase at the same rate as the control embryos. In fact the high EtOH groups heart rate increased at less than half the rate of the control group. It is interesting to note that higher EtOH exposed and control embryos had higher heart rates than lower EtOH exposed embryos. It is also interesting to note that the low EtOH group and the high EtOH group have slopes that do not differ by so much.

Figure 3: Vascular Area over Time



The above graph shows that control embryos developed larger vascular areas during every day, followed by 0.02 % EtOH exposed embryos and 0.002 % EtOH exposed embryos. The rates of growth (the slopes) were fairly uniform, with the high EtOH rate of vascular area development slightly lower than the other two. As we expected, the control group had the largest vascular areas over time.

Figure 4: Vascularization of a control chick embryo at day four.



The above image shows vascularization of a control chick embryo at day four. This photo was taken courtesy of Ashley Leslie and Diana Page. Vascularization is occuring as normal; blood vessels are developing. This image was observed with a Nikon Eclipse E200, and the image was photographed using a Sony DFW-X700.

The datum presented in these graphs relate to each other in a number of interesting ways. First of all, the two structures we measured, vascular area and body length, had rates of growth that did not deviate so much from group to group, whereas the slope of heart rate was very different in the control group than it was in the two EtOH exposed groups. Secondly, with the exception of vascular area, the quantities did not decrease directly with EtOH exposure. For example, the high EtOH group had higher body lengths over our given time span than our control group, which in turn had higher body lengths than the low EtOH group. Also, if a decrease in either of these quantities directly corresponded to ethanol concentration, then the low EtOH groups would have exhibited higher body lengths and heart rates than the high EtOH groups.

It should be noted that although the body length of the high EtOH group was higher than that of the control group, the control group's heart rate was faster by the end of the time span.

The mortality rates for all of the embryos were extremely high. No single embryo lasted for more than 6 days after the explant.

IV. Discussion and Conclusions

Our hypothesis that exposing the chicks to alcohol would decrease the area of the vascular area was supported; however, contrary to the hypothesis, heart rates in the control chicks were lower than heart rates in the chicks exposed to higher amounts of EtOH. Furthermore, our hypothesis that ethanol exposure would reduce body length was not supported; the high EtOH group had the highest body length to begin with, although it did not increase as rapidly as did the body length of the control group.

Many valuable conclusions may be drawn from the data. First, alcohol does not automatically cause a reduction in

heart rate. Although alcohol may interfere with the morphogenesis of the heart, it does not necessarily slow down its rate (Ruckman, 1988). Perhaps neural innervation caused the increased heart rates of the high EtOH group. I expected that, as malformations occurred in the atrium and the ventricle during developing stages, the blood would be unable to traverse the contours of the heart as effectively, and this would slow down the rate. However, when alcohol enters the blood of an animal it dehydrates the blood, which decreases the blood volume of the heart, making it speed up its rate (Erkboni 2004). The total volume of blood output may decrease as Bruyere found (Bruyere, 1988) but the heart rate was initially very high in embryos exposed to the higher concentration of EtOH. Furthermore, heart-rate is not dose-dependent on ethanol. The lower concentration of ethanol group exhibited a lower heart rate than the control group, showing there is no linear relationship between concentration of ethanol and heart rate. Perhaps the 0.02% ethanol exposure was enough to disrupt cardiogenesis but not enough to trigger a deydration of the blood significant enough to change its volume. Further experiments with several different ethanol concentrations ranging from 0.02% to 0.002% could reveal if there is a coherent relationship between these two quantities.

As hypothesized, vascular area decreased with ethanol concentration. There was not as much vascularization in ethanol exposed chicks. This was expected as alcohol interfers with retinol which is an important morphogen in vascular development. It would be wise in the future to conduct an experiment in which RA (retinolic acid) and alcohol were added to the same chick, to see if the RA offsets the negative effects of the alcohol. This could perhaps demonstrate that RA is an important morphogen in heart development.

One very interesting result was the size of the body length. Despite what was hypothesized chicks, which were exposed to the lower ethanol concentration, had larger body length than the controls. This shows that alcohol does not necessarily hinder the development of the chick embryo body, if it is in low concentrations. Furthermore, a reduced vascular size does not necessarily translate to a smaller embryo. These findings to not necessary contradict those of Yang (Yang, 2004). Perhaps alcohol does not itself interfere with body length, and that body lengths can develop in a lower vascular area, and in a smaller embryo. It is probable, therefore, that alcohol itself does not interfere with processes that increase body length. It is also possible that as heart rate begins to decrease in ethanol exposed chicks, their normal-length bodies are deprived of the amount of blood necessary to continue developing. A developing embryo requires nutriets and oxygen rich blood as it no longer receives these by diffusion. Potentially, we could measure the heart rate per body length ratio necessary for a developing embryo by doing another experiment, in which these two quantities are closely linked and mathematically analyzed. If more data is gathered from we could determine a heart rate necessary for a particular body size.

Our sources of error were many, as evidenced by the very high mortality rate of all embryos, ethanol-exposed and non-ethanol exposed. These sources could be due to a number of factors including leaving the embryo outside for too long a period, disturbing its normal temperature, mishandling it, or contaminating it by not applying proper sterile technique, although this last possibility is unlikely as no bacteria colonies were observed. In the future several more steps can be taken to reduce unnecessary error. Instead of carrying the embryos back and forth to the incubator we could wheel them on a cart, making the possibility of disturbing the embryo smaller. We could also devise a method to observe the embryo without opening the lid or culture the embryos in larger weigh boats (Patel, 2004).

Most important in any further experimentation on chicks would be to use a larger batch. There is no way to know if this data is accurate or whether it is given to fluke, as we had so little data to draw on. Increasing the amount of embryos explanted will not reduce the mortality rate, but it would equate to more embryos and more data. This data could be used in a number of exciting ways, some of which are mentioned above. Also, using greater ethanol concentrations would allow for better trend lines, which could give us more reliable trends to base further conclusions and hypothesis on.

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