I. Introduction

Zebrafish (Danio rerio) are a useful vertebrate model for the study of craniofacial development, which is a complex process that requires accurate formation of cartilage, bone, nerve, and muscle, occurring during embryogenesis. These organisms are externally fertilized, which allows for observation from the single cell stage, contributing to their status as exemplary model vertebrates for experimentation. Further advantages of the zebrafish embryos are their translucency, which enables examination of their development readily with a dissecting microscope. In addition, zebrafish have high fecundity (mature females lay several hundred eggs at weekly intervals), short generation time (3-4 months), rapid development, and easy laboratory maintenance (Browder and Iten, 1998).

Zebrafish development closely resembles that of higher vertebrates, as they form essentially all the same skeletal and muscle elements, which allows researchers to apply what they learn about zebrafish molecular signaling cascades, developmental abnormalities, and other experimental observations directly to humans (Payne-Ferreira et al, 2003). Zebrafish and similar vertebrates have become organisms integral to the advancement of scientific knowledge, as well as to the formation of medications and vaccinations, as the effects of chemicals on and the bodily processes and development of zebrafish reveal relevant information corresponding to human health. Consequently, research on zebrafish and similar organisms is invaluable, especially those involving the effects of mutagens such as nicotine, a chemical consumed by millions within the U.S. daily.

In early embryogenesis, there exist three distinct cell layers termed the endoderm, ectoderm and mesoderm. Through cell proliferation and signaling, as well as morphogenic movements, these layers re-arrange to form organs. Tetratogens, harmful substances that result in the development of abnormal body structures, can alter and inhibit these movements. While this experimentation did not directly test or observe molecular effects, aberrations in body structure of growing embryos, aberrations served as the manifestations of such effects. The extent and number of these defects in the tested organisms were viewed as indicators of the effects of the specific tetratogen being examined - Nicotine. Nicotine is a main ingredient in many tabacco products and is an identified tetratogen. Inhaled my millions each year,
Nicotine remains the cause of heart disease and respiratory complications. Specifically, experimentation has shown that Meckle's cartilage and the pharyngeal arches demonstrate severe deformity when exposed to Nicotine when exposed are compared to a control. Thus, in this study, I compared the width of the area between the distal bases of the 2nd pharyngeal arch of control zebrafish, and those exposed to Nicotine.

The pharyngeal arches in zebrafish are derived from neural crest cells, and begin to form within days of fertilization (Graham 253). There are a total of seven pharyngeal arches in zebrafish. Each arch is surrounded. As the various cells composing the arches must be co-ordinated such that all components of these structures form and the right time and place, interruptions in or impediments to the developmental process will most likely be reflected within these fragile cartilage base of the zebrafish embryos. Furthermore, these arches are also present in humans – a fact which allows our data to be extrapolated and applied to the ideas and research surrounding human anatomy. In humans, the pharyngeal arches form within the first 3- weeks of fertilization (Graham 253).

I have observed the effect of the concentration of nicotine upon these pharyngeal arches by subjecting zebrafsh embryos to various Nicotine solutions and then imaging and measuring the resulting body structures. Thus, in this study, I hypothesized that with an increase in nicotine content, a growth solution will be conducive to a proportional increase in developmental abnormalities and defects among the arches of the zebrafish embryos. As the 2nd pharyngeal arch is by far the largest and most prominent in addition to staining the darkest, I chose to measure the width of the area between the distal bases of this 2nd arch, expecting it to become smaller with an increase in Nicotine. Qualitatively, I expected the arches to be increasingly deviant in overall shape with more Nicotine present. By establishing a control consisting of a growth medium containing no nicotine, I compared and contrasted these developing cranial structures for size and general appearance among the embryos of the “pure” control and those of the mediums containing the various amounts of nicotine. Placing multiple embryos within each solution for experimental validity, I imaged and recorded all observations of the embryos at five days post fertilization.

II. Materials and Methods

This procedure was carried out with Jessica Gagnon on November 20, 2004 and the following procedural outline was written with her.

Four pairs of male and female zebrafish were crossed in order to collect fertilized eggs. A male and female zebrafish were placed together in a small tank to mate within their natural fresh water environment. The mating apparatus consists of one plastic tank with a small meshwork of holes at the bottom where eggs can fall through. This tank is then stacked atop an identical plastic tank to which it fits inside. This leaves a small space at the bottom, allowing a separation of the eggs from the mating pair, which remain in the upper tank. The mating apparatus was filled with water from the adult zebrafish tanks, as this ensured that the pH stayed at approximately 6.8 to 7.2, otherwise the fish will die. The apparatus was constructed in this way to separate the male and female pair from the shed eggs due to the fact that the fish could eat the eggs.

Because zebrafish mate on a light/dark cycle, they were placed together in a collection tank overnight in the dark, and collected in the morning after an automatic self-timer turned on the lights in the facility, thus allowing exposure to light. The zebrafish were collected in this manner because it is only in the light, after a prolonged period of darkness, that the zebrafish female is able to shed her eggs. This same procedure was done for each of the four pairs of zebrafish. Using a small fish net, the male was separated from the female in each of the four pairs, and the males and females were placed in separate tanks. At this point, the eggs were located within the bottom tank. They were isolated by pouring the water into a metal mesh net, through which the water would pass, but within which the eggs would remain. The eggs were then collected into a small Petri dish containing methylene blue, which is the embryo growth solution or fish water. The methylene blue growth solution was made by using one liter of water, then adding one teaspoon of salt and 20_l of methylene blue.

A 15mM stock solution of Nicotine tartrate was made by adding 0.346g of Nicotine tartrate to 50ml H2O. The pH of the stock solution was 3.8, which is too acidic due to the acidic nature of Nicotine tartrate. Zebrafish live at a pH ranging from 6.8 to 7.2; therefore, NaOH was added dropwise to the 15mM Nicotine stock solution until the pH was 6.8. The pH was measured by using a digital pH meter by Corning that produces a digital readout. Then the 1.0mM, 1.25mM, and 1.5mM solutions of Nicotine tartrate were made. The 1.0mM Nicotine solution was made by adding 666µl of 15mM stock to 9.334ml of fish water. The 1.25mM Nicotine solution was obtained by adding 833µl of 15mM stock to 9.334ml of fish water. The 1.25mM Nicotine solution was obtained by adding 833µl of
15mM stock to 9.167ml of fish water. Lastly, the 1.5mM Nicotine solution was made by adding 1ml of 15mM stock to 9ml of fish water.

The control consisted of 35 wild type embryos as the control organisms. There were 31 embryos in the 1.0mM and 1.25mM Nicotine solutions, and 29 embryos in the 1.5mM Nicotine solution. The embryos were left in these Nicotine solutions for 24 hours post fertilization. Then the Nicotine solution was pipetted out, fresh fish water was added to the Petri dishes, and the embryos developed until they were 5 days post fertilization.

At 5 days post fertilization, the embryos were put to sleep with the chemical called Tricaine. Then, the embryos were fixed in 4% paraformaldehyde for 6 hours. After 6 hours, the embryos were rinsed in a solution consisting of 60% ethanol and 40% phosphate buffered saline (PBT) for one hour to dehydrate, then were placed in Alcian Blue dye overnight, which resulted in the staining of the craniofacial cartilages. The PBT contained 0.1% Tween 20, which is a detergent used to prevent the embryos from sticking to each other. Next, the embryos were removed from the Alcian Blue stain and rehydrated in a solution of 50% ethanol/PBT for 30 minutes. Afterwards, the embryos were placed in 10ml PBT, then treated with 16mg/ml of Trypsin for 20 minutes at room temperature to remove excess tissue and to better visualize the stained cartilages. Lastly, the embryos were placed in 50% glycerol and 50% PBT to observe underneath the microscope.

At this point, the embryos were ready for analysis using a Zeiss Semi SVII stereoscope and the Axio Vision 3.1 imaging software. In order to image the embryos, the Axio Vision 3.1 software program was opened, the embryos were placed upon the microscope’s stage in their Petri dishes, and were focused under 66x magnification. The camera icon was clicked in the Axio Vision program and an image was taken. The entire craniofacial region was imaged including the Meckel’s cartilage and the 1 st pharyngeal arch. These two prominent structures were then scaled using Axio Vision by selecting the scale bar tool, clicking this icon, and dragging it to the desired position being measured. Then the images can be printed using a color printer. Since the scale bar tool only measures in predetermined increments, any precise measurements were determined by hand using a standard ruler and calibrating it to the computerized measurement on the imaged structure. Five individual embryos were chosen from the control, 1.0mM, 1.25mM, and 1.5mM Nicotine solution as representatives of the total population in each concentration. Images of the entire embryo were also taken to be used for qualitative analysis.

### III. Results

An increase in abnormalities was observed as the concentration of Nicotine increased. This was demonstrated in part by the quantitative data obtained, which revealed a general decrease in the width of the 2 nd pharyngeal arch with an increase in Nicotine concentration. However, this numerical data is not representative of the qualitative data observed from the images obtained, which in turn revealed what in some cases were severe defects in the shape of the structures of the fish, as well as misplaced and broken bones, among those exposed to the Nicotine. In the five day post fertilization zebrafish embryos, those embryos exposed to Nicotine showed deformities in their 2 nd pharyngeal arches which were particularly obvious when compared with images of the wild type fish. For example, some embryos within the 1.0 mM solution of Nicotine contained a 2 nd pharyngeal arch which had a much sharper area of attachment than those embryos of the control. Likewise, certain embryos of the 1.25 and 1.5-mM solutions contained arches which did not have a point of attachment at all – a severe mutation observed in multiple images taken through our experimentation. These mutations are illustrated in Figures 2-7. These qualitative mutations were also more prevalent in the higher concentrations of Nicotine. Also observed and shown in Figure 7, were mutations in many other parts of the embryo body, as the zebrafish placed in the 1.5 mM solution appears shrunken and contains many differences, all able to be seen in this sideways view of the embryos.
Figure 1: The cartilage of a 5-day post fertilization embryo not exposed to Nicotine

Figure 2: (above) The overall bodies of a wild type embryo (atop) and an embryo subjected to 1.5 mM Nicotine (bottom fish).

Figure 3: The cartilage of a 5-day post fertilization embryo after a 24-hour exposure to a 1.25-mM solution of Nicotine
The Effect of 24-hour Nicotine Exposure on Zebrafish Embryos

![Graph showing the effect of nicotine concentration on zebrafish embryos.](image)

**Fig. 1** Zebrafish embryos were exposed to nicotine tartrate, a known teratogen, for 24 hours post fertilization. Embryos were then placed in fresh growth solution to develop 5 days post fertilization and compared with wild type embryos. The average width of the area between the distal bases of the 2nd pharyngeal arches overall became smaller with an increase in the Nicotine concentration. However, it is important to note that size difference was not the only abnormality detected within the exposed zebrafish, as the figure does not reflect the qualitative differences observed. The width values shown in this figure represent the average of 5 zebrafish from each solution (n=5 for all 4 bars/concentrations).

![Image of cartilage from a 5-day post fertilization embryo after nicotine exposure.](image)

**Figure 5:** The cartilage of a 5-day post fertilization embryo after a 24-hour exposure to a 1.0-mM solution of Nicotine

![Image of cartilage from a 5-day post fertilization embryo subjected to 1.5 mM Nicotine.](image)

**Figure 6:** The cartilage of a 5-day post fertilization embryo subjected to a 1.5 mM Nicotine solution.
Figure 7: (Directly above) A representative image from each Nicotine solution is shown here. The 2nd pharyngeal arch is the triangular, dark blue stained structure within the embryo. Likewise, Meckle’s cartilage can be observed in these photos: this structure is the circular shaped, most anterior structure shown in the images. The straight black line along the image of the wild type embryo denotes the area which was measured in these organisms for this experiment – the width of the area between the distal bases of the 2nd pharyngeal arch. From these pictures, it is apparent that both the 2nd pharyngeal arch and Meckle’s cartilage deviate from the standard size and shape of the wild type embryos.

IV. Discussion and Conclusions

The experimental data supported my main hypothesis, which stated that with an increase in Nicotine concentration, an increase in the amount and severity of abnormalities and defects of the craniofacial structures, specifically the 2nd pharyngeal arch, would also be observed. However, there was not a linear decrease in the width of the space between the distal bases of the arches with an increase in Nicotine, as was expected. While this measurement did in fact decrease on average from the wild type embryos in the 1.0 mM and 1.25 mM, this was not so in the 1.5 concentrations. While the embryos of the 1.5 mM solution did in fact appear, upon close examination, the most deformed in relation to the wild type embryos, the quantitative data indicated a less severe difference. Out of alignment with the other arches with a portion of the cartilage even missing in an area, Figure 4 depicts the level of severe mutation in one of the observed 1.5 mM embryos, even as the first figure does not graphically reflect this aspect of the image. Thus, while the qualitative portion of my hypothesis was supported with the increasingly severe degree of the mutations of the 2nd pharyngeal arch, these abnormalities did not necessarily manifest themselves in a decrease in size, thus refuting the quantitative part of my hypothesis. While some mutations involved a flattening of the Meckle’s cartilage above the arches, the width at the arch base consequently widened, resulting in the observed slight increase in the width of the base from the 1.25 mM to 1.5 mM solution embryos.

Likewise, a parallel experiment carried out by my lab partner, Jessica Gagnon, produced similar results. Carrying out the same procedure as described above in the Materials and Methods section, Jessica’s work differed only in her measurement of the Meckel’s cartilage instead of the width of the 2nd pharyngeal arch. However, as is also apparent from the images above, the Meckel’s cartilage exhibited mutation severity proportional to the Nicotine concentration. While this cartilage flattened, sharpened, or elongated, depending upon the embryo, prominent differences were in fact observed, and once again, were not necessarily represented by linear quantitative results.

Although there were relatively few sources of error in this experiment, an assumption based on the correlation of the results to the hypothesis and previously published research, certain aspects can be improved and refined. For example, due to the fragility and tiny size of the embryos and their cartilage structures, upon their transference to a microscope slide after 5 days, some structures were undoubtedly broken and torn, not as a direct result of the Nicotine effects but because of our technique and instruments. Should this have happened, the validity of our results would be significantly lowered. For future experimentation, new techniques could be studied and more efficient tools obtained, while practice trials could also be done to refine techniques and encourage more accurate results.

Furthermore, should the number of embryos of each concentration averaged for data, validity would also be increased. While only five embryos from each solution were taken into account for each determined average in Figure 1, more were available, the only constraint being time. Thus, had ten or fifteen embryos for each concentration been averaged, a larger percentage of the overall population of the zebrafish would have been surveyed and taken into account, making the overall data more reliable.

Although successful in the production of adequate and fairly reliable data, our experimentation leaves much room for further research. For example, other craniofacial structures of the embryos could be focused upon for measurements, such as the neurocranium. Also, the entire body of the zebrafish embryos could be measured in an effort to recognize
any overall patterns in size. Furthermore, while both sets of structures tested in our experimentation, the pharyngeal arches and Meckel’s cartilage, are neural crest cell derived, other such structures could be measured and imaged to determine if all neural crest cell derived structures are susceptible to deformation from Nicotine. Should these structures all exhibit particularly severe defects as a result of the Nicotine, certain conclusions could then be made regarding the exceptional susceptibility of neural crest cells to this known carcinogen and tetratogen. As little research has been done regarding this particular area of research, the knowledge that could be potentially gleaned from such work would be cutting-edge and new to the science world. With millions of humans becoming addicted to Nicotine each year and dying directly from smoking related complications, the information that could be obtained from further study in this area is invaluable.

V. Bibliography


