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Effect of Arsenic Exposure on Early Eye Development in Zebrafish (Danio rerio)

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THE EFFECT OF ARSENIC EXPOSURE ON EARLY EYE DEVELOPMENT IN ZEBRAFISH (*Danio rerio*)

By

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An Abstract of the Thesis Presented
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Arsenic is a metalloid that contaminates drinking water supplies worldwide. Due to concerns for human health, the World Health Organization (WHO) and the U.S. Environmental Protection Agency (EPA) have established a safe level in drinking water of ≤ 10 ppb. Arsenic has been shown to have carcinogenic effects in humans at high and low doses. Chronic exposure may result in dermal conditions such as hyperkeratosis and hyperpigmentation. Recently, arsenic exposure has also been linked to lower IQ values in children. The effect of arsenic on neurogenesis, specifically eye development, has not been widely explored. This study aimed to examine the effect of environmentally relevant concentrations of arsenic on early eye development by morphological and molecular analysis.

The zebrafish (*Danio rerio*) was chosen to model the impacts of arsenic on retinogenesis because of its similarities to human eye development both structurally and genetically. The effect of arsenic exposure on the gross morphology of the eye and tissue development via histology were examined at three larval stages. It was found that exposure to arsenic has an effect on eye morphology resulting in a significant increase in eye diameter at 14 dpf (days post

fertilization) relative to control under all treatment conditions. This was coupled with a trend in thinning of the retinal pigmented epithelium (RPE) layer in fish exposed to 500 ppb arsenic.

The impacts of arsenic exposure on gene expression were analyzed following treatments of 10, 50, and 500 ppb from 1 – 72 hpf (hours post fertilization). Molecular analysis of genes associated with eye development was investigated by RT-qPCR at 32 and 48 hpf. RT-qPCR results revealed differential expression of *Pax6a*, *Pax2a*, *Ngn1*, *Sox2*, and *Shha* relative to control. Specifically *Pax6a*, *Pax2a*, and *Sox2* are found to be important in the formation of the RPE. Proper formation of the RPE is necessary for growth of the sclera which, in turn, is responsible for maintaining the shape of the eye. Although we observed a thinning of the RPE we also noted an overall increase in eye size of 14 dpf zebrafish. This could potentially be explained by the disruption of gene expression under arsenic exposure during critical time points in early eye development.

ACKNOWLEDGEMENTS

First I would like to give my immense gratitude to my advisor Rebecca Van Beneden. Upon joining the lab Becky gave me the opportunity to pick and design my own project and supported me through its completion. This allowed me to choose something I was passionate about and for that I will be forever grateful. Her guidance and encouragement were a critical part to my success and I am excited to take everything that she has taught me and use it in my career as a Scientist. I would also like to give many thanks to my committee, Drs. Julie Gosse and Robert Wheeler. Their advice and excitement around science propelled me through my project and their insight played a key role in my experimental design and analysis. I would like to thank University of Maine Zebrafish Facility and specifically Mark Nilan for his dedication and care to providing the zebrafish used in this project. To my lab mate, Torey Bowser, I thank you for your continued support and friendship over the years. To Dr. Heather Hamlin, thank you for the use of your histology equipment and Dr. Leeanne Thayer for your help and expertise in histology. To the University of Maine Animal Health Lab, I thank you for your fantastic work in histological staining. To Nitya Murthy, I thank you for your long time friendship, witty intellect, and critical review of this thesis as an optometrist. To my friends and family, I thank you for cheering me on and encouraging me throughout my graduate career. To my parents, for raising me in an environment that encouraged curiosity and being in nature, you are the reason for my love of the biological sciences. To David, thank you for being my support system at home, listening to my ups and downs, and giving me confidence to push through Master's thesis. I couldn't have done this without you!

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CHAPTER 1

BACKGROUND

1.1 Environmental Arsenic Contamination

Arsenic is considered one of the World Health Organization's (WHO's) top 10 chemicals of concern for public health. Arsenic can enter the environment naturally through bedrock aquifers and volcanic off gassing as well as anthropogenically via industry and fertilizers (Figure 1.1). Two forms of arsenic exist in the environment, organic and inorganic. Organic forms, which tend to be byproducts of plants and animals, contain at least one carbon atom. Monomethylarsonoic acid (MMA) and dimethylarsinic acid (DMA) are often found in fruits, vegetables, and grains. Arsenocholine and arsenobetaine are organic arsenicals commonly found in seafood. Inorganic forms occur when arsenic, found in the environment, binds to sulfur, oxygen, or chlorine (ATSDR, 2011; Figure 1.2). Although many pesticides containing inorganic arsenic such as lead arsenate (apple pesticide) have been banned in the U.S., production of chromated copper arsenate (CCA) still continues (Hughes et al. 2011). CCA is used as a wood preservative in nonresidential facilities and is a current source of environmental arsenic pollution. Inorganic arsenic contamination affects drinking water supplies worldwide. Both the WHO and the U.S. Environmental Protection Agency (EPA) have established a safe drinking water level of less than or equal to 10 µg/L (10 ppb). However, in many countries, such as Bangladesh and Chile, arsenic in drinking water supplies has been detected at levels up to 2,500 and 800 µg/L, respectively (Naujokas et al. 2013). Arsenic levels in drinking water in regions of the United States may also exceed safe levels, although the upper range generally lies between 100 and 500 µg/L. Arsenic is predicted to exceed 10 µg/L in basin-filled aquifers in the southwestern U.S. (*i.e.*, Arizona and Utah; Anning et al. 2012) and has been found to exceed the

safe limit in Nevada (Thundiyil et al. 2006), North Carolina (Sanders et al. 2012), and throughout New England (Ayotte et al. 2003; Nielson et al. 2010). Private well owners are especially at risk for arsenic contamination as there are currently no regulations that require testing. In the Northeastern United States private wells can contain arsenic concentrations greater than 50 µg/L (Focazio et al. 2000). Although arsenic treatment systems can be implemented, they are expensive and may fail if not maintained properly. Flanagan et al. (2015) showed a 15% failure rate in arsenic treatment systems in central Maine, suggesting that consumption of arsenic-contaminated well water may occur even if preventative actions have been taken.

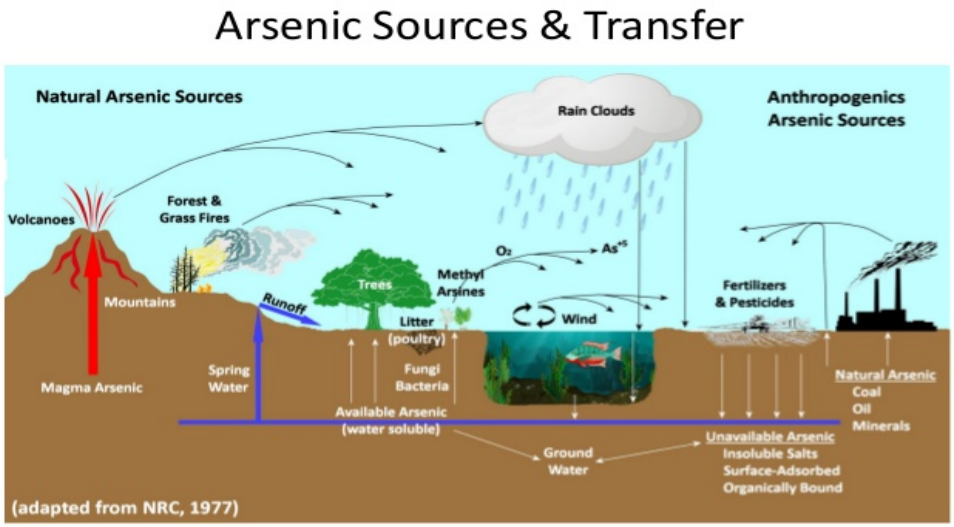

Figure 1.1: Arsenic sources of transfer. Natural and manmade sources of arsenic in the environment. Retrieved from https://www.slideshare.net/MSTomlinson/oahu-soil-sediment-as, adapted from NRC, 1977.

1.2 When Humans are Exposed to Arsenic: Arsenic Metabolism

Humans are most likely exposed to inorganic arsenic in drinking water, however they may also be exposed by consuming arsenic contaminated foods. Some forms of dietary organic arsenic, such as aresnobentaine in fish (Li et al. 2017), are not known to be toxic to humans. More toxic inorganic forms are found to accumulate in rice and seaweed and are found to be at similar concentrations compared to drinking water (Brandon et al. 2014). Arsenic in drinking water is commonly in the form of sodium arsenite ($AsNaO_2$; Figure 1.3A). Sodium arsenite (also known as sodium-meta arsenite) is the sodium salt of arsenous acid and disassociates upon entrance to the cell (Escudero-Lourdes 2016). The trivalent form of arsenic (AsIII) enters the cell primarily through aquaglyceroporins (Drobna et al. 2010). The pentavalent form of arsenic, sodium arsenate (Figure 1.3B), which also contaminates surface waters, enters the cell via phosphate transporters. Upon entry into the cell, sodium arsenate is reduced to AsIII. AsIII is conjugated to glutathione and is then methylated resulting in dimethylated or monomethylated forms (Watanabe and Hirano 2013). At this time it can be excreted from the body via urine or feces, but much of it is stored in tissues such as the kidney, lung, heart, and brain (Leslie et al. 2004). Kleiman et al. (2016) showed that chronic exposure to 10 ppm $AsNaO_2$ resulted in arsenic accumulation in the eye (lens) that was significantly higher than µg arsenic in the lung, liver, heart, and brain at 4 weeks and 6 months of continuous exposure in male mice. A similar study was conducted in zebrafish, in which there was an elevated accumulation of arsenic in the eye at 100 and 300 ppb treatment conditions compared to brain, gill, and muscle at 6 months of chronic exposure (Hallauer et al. 2016).

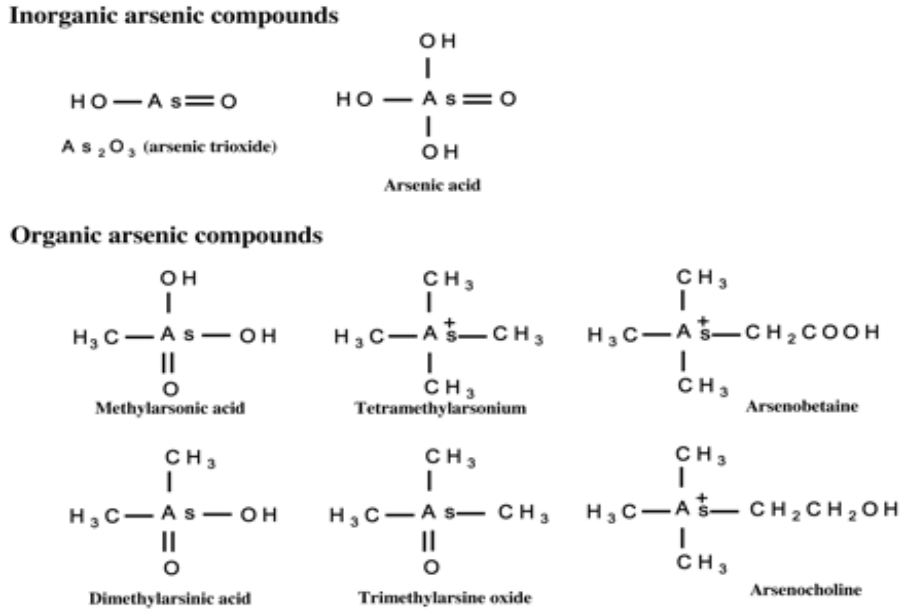

Figure 1.2: Structures of inorganic and organic arsenic compounds (Hikita et al. 2011).

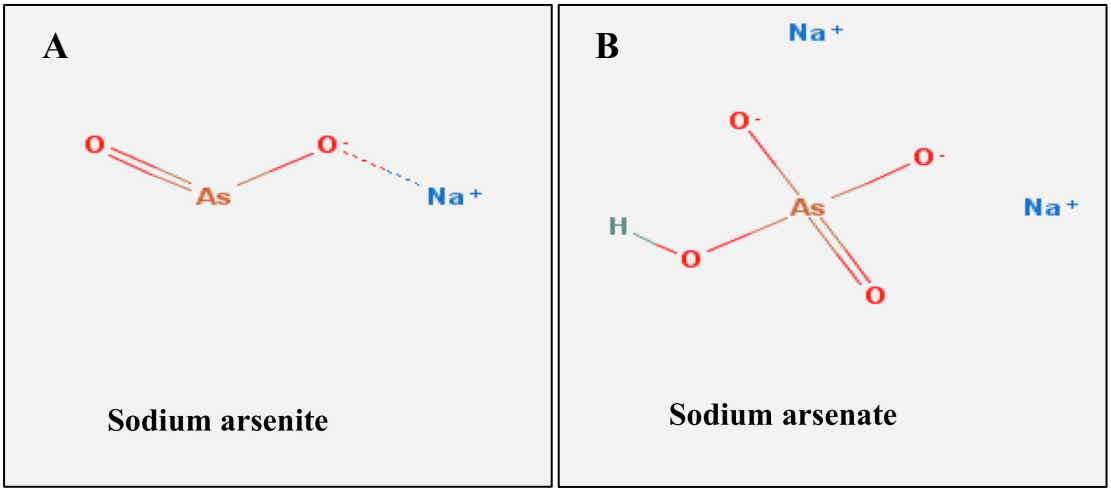

Figure 1.3: Structures of common inorganic arsenic compounds in water supplies. Sodium arsenite (A) and sodium arsenate (B), retrieved from https://pubchem.ncbi.nlm.nih.gov.

1.2.1 The Impacts of Arsenic on Human Health

Chronic exposure to high levels of arsenic has been linked to bladder, lung, and skin cancer (Smith et al. 2006; Applebaum et al. 2007; Baris et al. 2016) as well as neuropathy, diabetes**,** liver disorders, and cardiovascular disease (Liu and Waalkes, 2008; Kundu et al. 2011; Gong and O'Bryant, 2012; Bräuner et al. 2014). Most studies have focused on how arsenic may

impact adults in contaminated regions but there has been growing interest in understanding how arsenic is affecting early childhood development. A meta-analysis of data collected from 2000-2012 revealed neurological deficits in children residing in areas with arsenic levels ranging from 120 to 470 ppb found in drinking supplies (Rodriguez-Barranco et al. 2013). A comparison was made between the level of arsenic in the drinking water and the percent arsenic detected in urine, to determine potential neurological damage in children ages 5 – 15. It was shown that an increase of arsenic above the safe limit in drinking water was correlated with an increase of arsenic in urine samples which correlated with a decreased intelligence quotient (Rodriguez-Barranco et al. 2013). More recently, studies have been conducted on the transplacental transfer of arsenic from the mother to her fetus during pregnancy and have associated low levels of arsenic with effects on fetal growth (Gilbert-Diamond et al. 2016). Similar laboratory studies have also been executed on mice, in which transplacental transfer of arsenic to the fetus resulted in increased tumor prevalence in male offspring at 2 years of age (Tokar et al. 2012).

1.2.2 Impacts of Arsenic on the Visual System

There is increasing evidence that arsenic may have a widespread negative impact on neurogenesis during early development but the effects of arsenic on the ocular system specifically have yet to be determined. Banerjee et al. (2011) showed that individuals with specific genotypes were more susceptible to arsenic-induced ocular diseases, but there has been little other research exploring the impact of arsenic on the developing visual system. A study done in southwestern Taiwan showed evidence that arsenic exposure is associated with the development of pterygium, a condition that can lead to blindness by outgrowth of the surface membrane of the eye (Lin et al. 2008).

1.3 Retinogenesis and Genetic Regulators

Retinogenesis requires a complex network of developmental genes to be expressed at specific times to induce formation of the optic cup, differentiation of the neural retina from retinal pigmented epithelium (RPE), and generation of retinal vasculature and maturation of the optic nerve (Malicki et al. 2016). One way to analyze development of the visual system is to track changes in genes critical for successful eye development and neurogenesis**.** One such gene, *Pax6*, acts as a transcription factor with two DNA binding domains, a homeodomain and paired domain (Figure 1.3) allowing it to regulate a number of genes critical for eye development (Haubst et al. 2004).

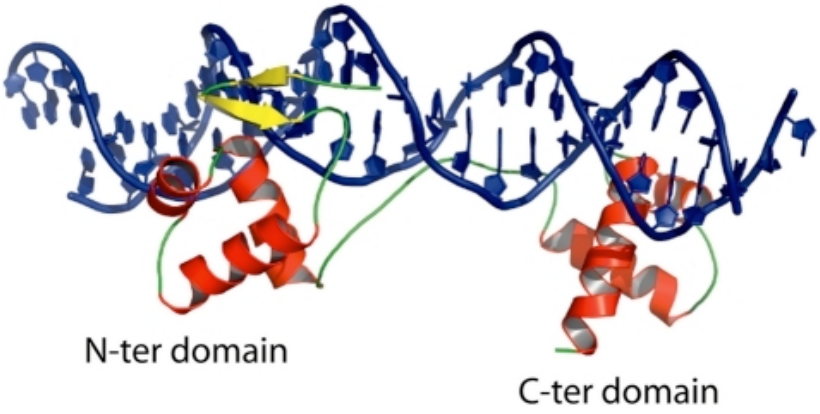

Figure 1.4: Pax6 protein structure. Paired domain (N-ter domain) and homeodomain (C-ter domain) interacting with DNA to regulate gene expression (Alibes et al. 2010).

Pax6 is highly conserved and is essential for the development of a functional eye, specifically lens formation and neural retina organization. Pax6 works closely with Pax2 to define RPE boundaries (Ashery-Padan et al. 2000). In most cases, humans heterozygous for the functional loss of one *Pax6* allele develop aniridia, a genetic disorder characterized by small iris size, impaired vision, and the onset of glaucoma (Hingorani et al. 2012). *Pax6* mutations in mice and rats cause micropthalmia, or small eye size, and a phenotype analogous to that of human aniridia (Hill et al. 1991). Because of its early embryonic involvement in eye, brain, and spinal cord

formation *Pax6* is critical for survival. Homozygous loss of *Pax6* results in loss of functional eyes, CNS abnormalities and is typically fatal before or soon after birth (Hogan et al. 1986; Glaser et al. 1994). By determining the expression of *Pax6*, along with genes that are located up- and down-stream in the ocular development pathway, such as *Pax2*, *Shh*, *Sox2*, *Ngn1* and *Six3b* (ortholog to *Six6*), we can gain insight on how arsenic may be affecting eye development on a molecular level (Figure 3.1A).

1.4 Using the Zebrafish to Model Human Retinogenesis

This study used the zebrafish, *Danio rerio*, as a model organism to investigate how environmentally relevant levels of arsenic may be affecting eye development. The zebrafish, along with other fish species, have shown neurological deficits when exposed to arsenic. These include facial and cranial malformations, deficits in sensory-motor response (Li et al. 2009), and alterations in swimming patterns (Baldissarelli et al. 2012). Zebrafish have also become useful in modeling vertebrate retinal disease because of their rapid eye development and structural and functional similarities to the mammalian retina (Gestri et al. 2012). The first morphological event in zebrafish eye development is the formation of the optic lobe (optic vesicle) from primordial CNS at 11.5 hours post fertilization (hpf). The RPE first becomes distinguished at 15 hpf and continues development through 48 hpf. Subsequently, the optic lobe transforms into the optic cup and the lens placode becomes more pronounced. At 48 hpf, ganglion cells and other retinal neurons begin differentiating and are post mitotic by 60 hpf. Developing retinal cell layers include the following: ganglion cell layer (ganglion cells), inner plexiform layer (amacrine cells), inner nuclear layer (horizontal & bipolar cells), and photoreceptor layer (rods and cones; Figure 1.3).

Although retinogenesis is mostly complete by 60 hpf and the zebrafish is hatched by 72 hpf (dependent upon temperature), the eye still grows in size throughout development and neurogenesis continues throughout the organism's life span (Malicki et al. 2016).

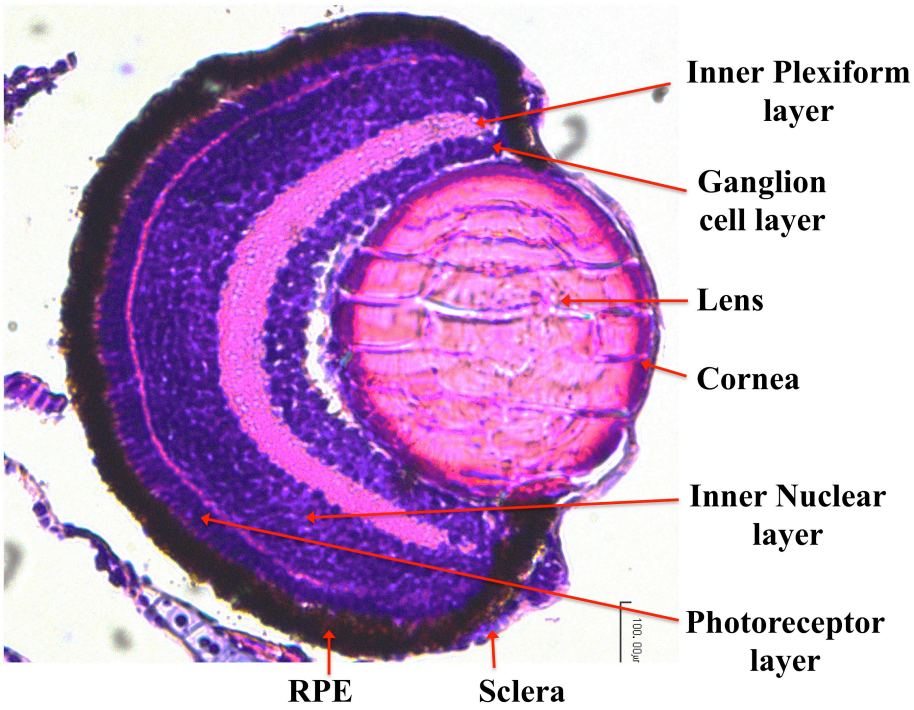

Figure 1.5: Anatomy of the zebrafish retina. Hematoxylin and eosin (H & E) stained 6 µM axial section of zebrafish retina at 14dpf with labeled layers of the retina. From outer to inner retina: ganglion cell layer, inner plexiform layer, inner nuclear layer, photoreceptor layer, RPE. The sclera, cornea, and lens (important components of the eye) are also labeled for visualization. Photo by R. Babich.

1.5 Summary

Arsenic exposure has been detected at levels that exceed the safe drinking standard of 10 ppb in water supplies worldwide. Given that many individuals are exposed to concentrations of arsenic exceeding 10 ppb throughout their lifetime, it is important to understand how arsenic may be impacting early development. Epidemiological studies have been conducted and there is a growing awareness that early exposure to arsenic may be resulting in decreased intelligence

quotients in children. How arsenic may be impacting neurogenesis on a molecular level has yet to be determined. Very few studies have been conducted on the impacts arsenic may have on the visual system specifically. Successful development of the visual system requires a complex network of genes, including paired box genes *Pax6* and *Pax2*, and is critical to a functional lifestyle. The zebrafish, which shares functional and structural similarity with human eyes, offers the ability to model the effects of arsenic on the visual system. This Master's Thesis aims to use the zebrafish model to identify changes in the expression of genes associated with eye development during neurogenesis correlated with the morphological consequences and impacts on retinal tissue development. The results of this study could be extrapolated to give insight into the effect of arsenic on neurogenesis and early development of humans living in areas with contaminated drinking water supplies.

CHAPTER 2

IDENTIFYING THE IMPACTS OF ARSENIC ON RETINOGENESIS

2.1 Materials and Methods

2.1.1 Fish Care and Exposure

One-cell AB-strain zebrafish embryos (University of Maine Zebrafish Facility) were treated with 0, 10, 50 or 500 ppb (500 µg/L) $AsNaO_2$ (Sigma Aldrich, St. Louis, MO) in egg water (60 µg/mL salt concentration made from Instant Ocean ®, St. Blacksburg, VA in D.I water). The treatment regimen was chosen based upon the following: 10 ppb arsenic treatment is the current EPA safe drinking water limit, 50 ppb was the previous EPA limit (10 ppb regulations were enforced in 2006), and 500 ppb is at the upper level found in private wells in the U.S. All treatments are environmentally relevant. Approximately 500 embryos were maintained in groups of 20 in a 28.5°C, 14:10 light -dark cycle incubator with 2.5 mL of solution per embryo in 50mL petri dishes for RNA extractions at 32 and 48 hpf. Approximately 200 embryos were maintained in groups of 20 in a 28.5°C, 14:10 light -dark cycle incubator with 2.5 mL of solution per embryo in 50mL petri dishes for morphological and histological analysis. Throughout arsenic exposure the chorion was left on (until a natural hatch between 48 and 72 hpf) so that the study was more biologically relevant. Although the chorion has been seen to hinder the effects of arsenic reaching the embryo, arsenic is still able to pass through (Olivares, et al. 2016). Following hatch (~72 hpf), larvae were moved to 200 mL clean egg water at room temperature at a density of 50 fish/250mL beaker with 50% water changes every two days. Fish were fed ground Tetrafin flakes until sacrificed. This protocol was approved by the University of Maine IACUC, protocol number A2017-05-04.

2.1.2 Morphological and Histological Analysis

Fish were euthanized with buffered 0.025% MS-222 at 4, 7, and 14 days post fertilization (dpf), fixed in 10% buffered formalin for 24 hours, then transferred to 70% ethanol. Zebrafish were imaged under a Nikon P-DSL32 dissecting scope using a Nikon DS-Fi2 digital camera and examined for head and eye malformations. Eye diameters were measured and compared to those of controls using ImageJ software. A randomized subset of 6 fish per treatment and stage was used for histology. Tissues were dehydrated using an ethanol bath series to 100% citrosolv and embedded in paraffin (Freitag et al. 2016). The following modifications were used to orient the eyes for uniform sectioning. Fish were cured in paraffin overnight and extracted after 24 hours. This extra step, to allow the fish to harden in paraffin overnight, stabilized the larval body, which enabled more consistent positioning in paraffin in step 2. Extracted fish were then re-embedded in paraffin in proper orientation for uniform eye sectioning. Serial sections of 6 μm were taken of the eyes, stained with hematoxylin and eosin (University of Maine Animal Health Lab) and analyzed using ImageJ software to measure retinal cell layer thickness.

2.1.3 RNA Extraction and Quantitative Expression using RT-qPCR

Embryos were collected from exposure solutions at 32 and 48 hpf, mechanically dechorinated, and homogenized. RNA was extracted from homogenates using Qiashredders and RNEasy minikit (Qiagen, Valencia, CA) following manufacturer's protocol. In each biological replicate there consisted 10 whole embryos per each RNA extraction. A total of 6 extractions were done per treatment and stage (60 embryos). cDNA was synthesized using an iScript cDNA synthesis kit and used for RT-qPCR (BioRad, Hercules, CA). Genes of interest, *Pax6a*, *Pax2a*, *Shha*, *Six3b*, *Sox2*, *Ngn1*, and *Ascl1a,* were amplified using iTaq Universal SYBR green reagents (BioRad, Hercules, CA). Efficiency was optimized between 90% - 110%. Amplification

procedures were completed using a CFX96 real time thermocycler (Biorad, Hercules, CA). See Appendix for thermocycler protocol details (Table A1) and primer sequences (Table A2); primers were synthesized by Integrated DNA Technologies (Skokie, IL).

2.1.4 Statistical Analysis

qPCR target genes were normalized to *B-actin* expression and fold change was calculated using the Pfaffl method (Pfaffl 2001). A one-way ANOVA was performed to test for significance between treatments at 32 and 48 hours post fertilization (hpf). Levene's test was used to determine variance homogeneity and Shapiro-Wilke was used to determine if data were normally distributed. Significance was confirmed using post-hoc Tukey's test. Upon failure of Levene's test, Games and Howell post-hoc was used to determine significance among treatments. Linear regression analysis was used to test the affect of $AsNaO_2$ treatment on peripheral and posterior RPE thickness. Statistical tests were conducted using SPSS software (IBM, Armonk, NY). A one-way ANOVA was also used to test for significance between treatments at 4, 7, and 14 dpf for morphological and histological analysis.

2.2 Results

2.2.1 Morphological Analysis

At 4, 7, and 14 dpf, arsenic-exposed larvae were analyzed for alterations in eye size (Figure 2.1C). At 14 dpf all three treatment levels resulted in a significantly increased horizontal eye diameter compared to the control (Figure 2.1A). Fish exposed to 10 and 500 ppb showed a significantly larger vertical eye diameter relative to control (Figure 2.1B). Fish from 50 ppb treatments had a slightly increased vertical diameter relative to controls but data were not significant (Figure 2.1B).

Horizontal to vertical ratios were calculated to distinguish if eye shape was round or oblong. There was no significant effect of treatment on the ratios (data not shown).

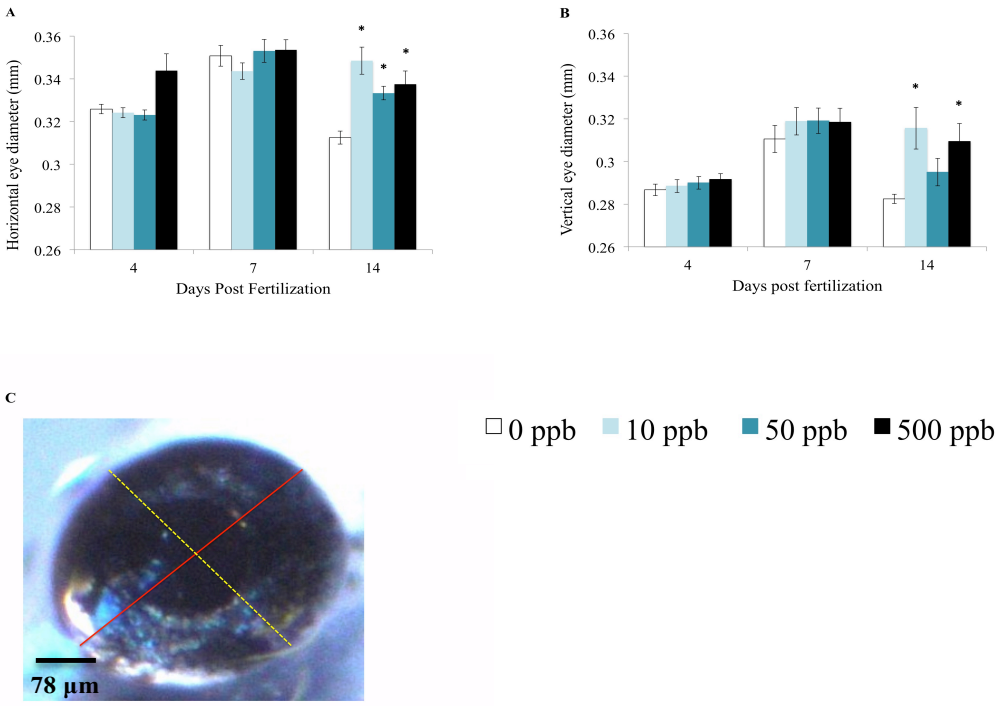

Figure 2.1: Morphological analysis. (A) Horizontal and (B) vertical measurements of zebrafish eyes under 0, 10, 50, and 500 ppb $AsNaO_2$ treatments from 1 – 72 hpf at 4, 7, and 14 dpf. Bars represent length measured in mm ± SEM. * $p \leq 0.05$ from respective control, see Appendix Table A3 for specific p values. (C) Measurements were taken by imaging individual zebrafish eyes and using ImageJ software to calculate both horizontal (solid line) and vertical (dashed line) diameters.

2.2.2 Histological Analysis

Following morphological analysis, whole zebrafish were sectioned and eye tissue was analyzed histologically. The ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform and photoreceptor layers, and RPE were measured axially using ImageJ software (Figure 2.2). There was a trend of reduced RPE cell layer thickness, both peripherally (near the lens) and posteriorly (mid-back of the eye), from 7 to 14 dpf at 500 ppb $AsNaO_2$ treatments. A linear regression was calculated to predict if RPE thickness is affected by $AsNaO_2$ treatment. A

significant regression equation was found ($F(1,21) = 7.390$, $p = 0.013$), with an R^2 of 0.260. Posterior RPE thickness decreased at 14 dpf as treatment increased ($\beta = -0.005$). Controls, 10 ppb, and 50 ppb treatments show a pattern of increasing posterior RPE thickness from 4 to 14 dpf (Figure 2.3B). This pattern is also seen in controls for peripheral RPE thickness (Figure 2.3A). In 10 and 50 ppb treatments there is a slight decrease in peripheral RPE thickness at 7 dpf (not significant) followed by an increase in thickness at 14 dpf (Figure 2.3A).

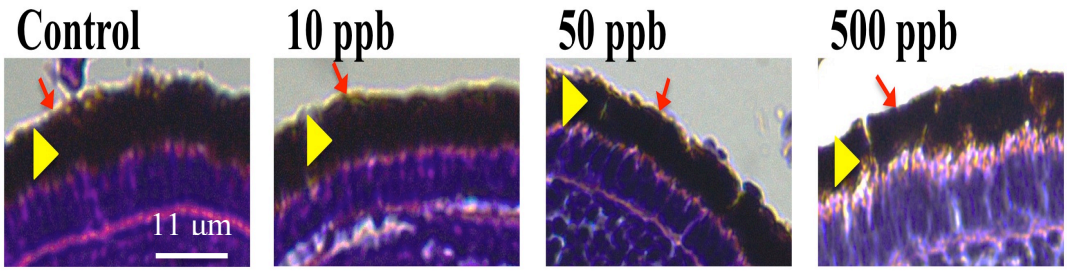

Figure 2.2: H & E stained sections of the zebrafish eye. Shown at 14 dpf cropped to emphasize differences in RPE thickness layer (yellow triangle) under $AsNaO_2$ treatments. Sclera/choroid location indicated by red arrow. The ne

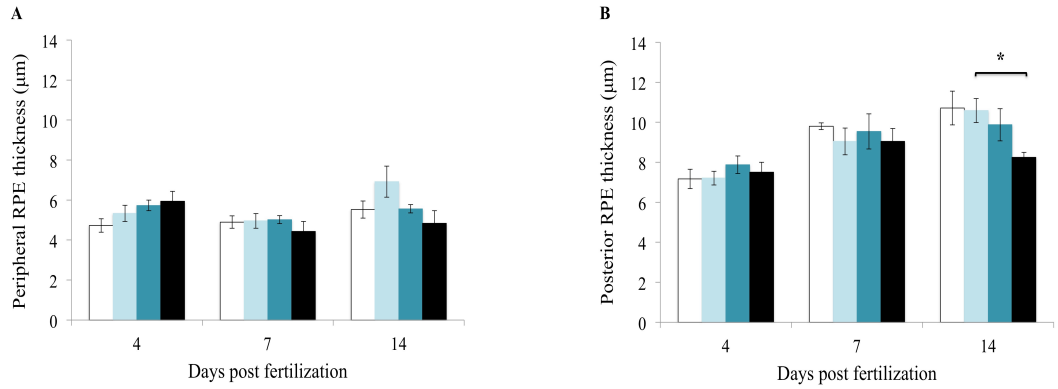

Figure 2.3: Histological analysis. (A) Peripheral and (B) posterior RPE cell layer thickness under 0, 10, 50, and 500 ppb $AsNaO_2$ treatments from 1 – 72 hpf at 4, 7, and 14 dpf. Bars represent cell layer thickness measured in µm ± SEM. * $p \leq 0.05$ from respective control. See Appendix Table A3 for specific p values. There was a significant regression in the posterior RPE thickness at 14 dpf with a $p = 0.013$

2.2.3 Quantitative Analysis of Genes Associated with Eye Development and Neurogenesis

Seven genes were analyzed by RT-qPCR for potential alterations in expression in response to $AsNaO_2$ exposure. *Pax6a*, *Pax2a*, *Shha*, *Sox2*, *Ngn1*, *Six3b*, and *Ascl1a* were chosen because of their prominent roles in early eye development and neurogenesis (Pébay et al. 2014). These genes are also shown to interact during neurogenesis (Figure 2.4) as determined by Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, CA; see Appendix Figure A1-2). Gene expression was analyzed at 32 and 48 hpf as these are critical time points in zebrafish in the development of the neural retina and the RPE. Arsenic has also been seen to have a previous effect on mRNA expression levels of Sox2, Pax6, Ascl1, and Shha (Al – Eryani et al. 2012, Tyler and Allan 2013, Fei et al. 2010).

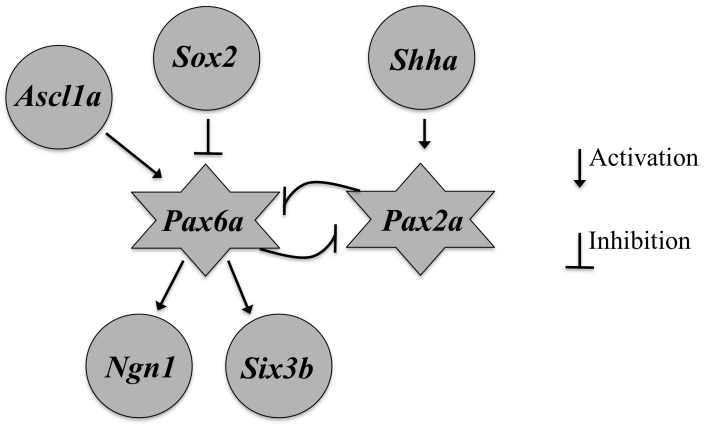

Figure 2.4: Interactions between genes of interest during neurogenesis. Pathway modified from Ingenuity Pathway Analysis software (Qiagen Bioinformatics, Redwood City, CA).

Pax6a, Pax2a, *Shha*, *Sox2,* and *Ngn1* were differentially expressed among treatments at both 32 and 48 hpf (Figure 2.5A-E). *Six3b* showed trends in expression among treatments but

data were not statistically significant (Figure 2.5F). *Ascl1a* expression was differentially expressed at 32 hpf but shows little variation from control expression levels at 48 hpf (Fig. 2.5G).

Pax6a, *Ngn1*, *Shha*, and *Pax2a* were significantly upregulated with 50 ppb $AsNaO_2$ treatment at 32 hpf (Figure 2.5A-D). *Ngn1* and *Pax2a* were significantly upregulated under 50 ppb $AsNaO_2$ treatments (Fig. 2.5B-C) at 48 hpf and *Sox2* was significantly upregulated under 500 ppb $AsNaO_2$ (Figure 2.5E). At 500 ppb arsenic exposure *Pax6a, Pax2a*, *Shha*, and *Ascl1a* expression at both 32 and 48 hpf were downregulated relative to control (Figure 2.5A,B,D,G). A similar downregulation occurs under 10 ppb treatments at 32 and 48 hpf of *Pax6a*, *Pax2a*, *Six3b*, and *Ascl1a* (Figure 2.5A,B,F-G). There was increased expression at 32 and 48 hpf under 50 ppb treatment of *Six3b*, *Shha*, *Sox2*, *Ngn1*, and *Pax2a* (Figure 2.5B-F). The greatest effect of $AsNaO_2$ on gene expression occurred in fish exposed to 50 ppb arsenic at both 32 and 48 hpf. See appendix Figure A4 and A5 for further visualization of gene expression data by heat map clustering. We have noted that at 50 ppb there is a lack of correlation between the gene expression data and alterations in morphology. Previous studies have suggested that arsenic may go through a different toxicity mechanism at high and low doses (Davey et al. 2007, Bodwell et al. 2004), which could possibly explain the gene expression irregularity seen at 50 ppb treatments.

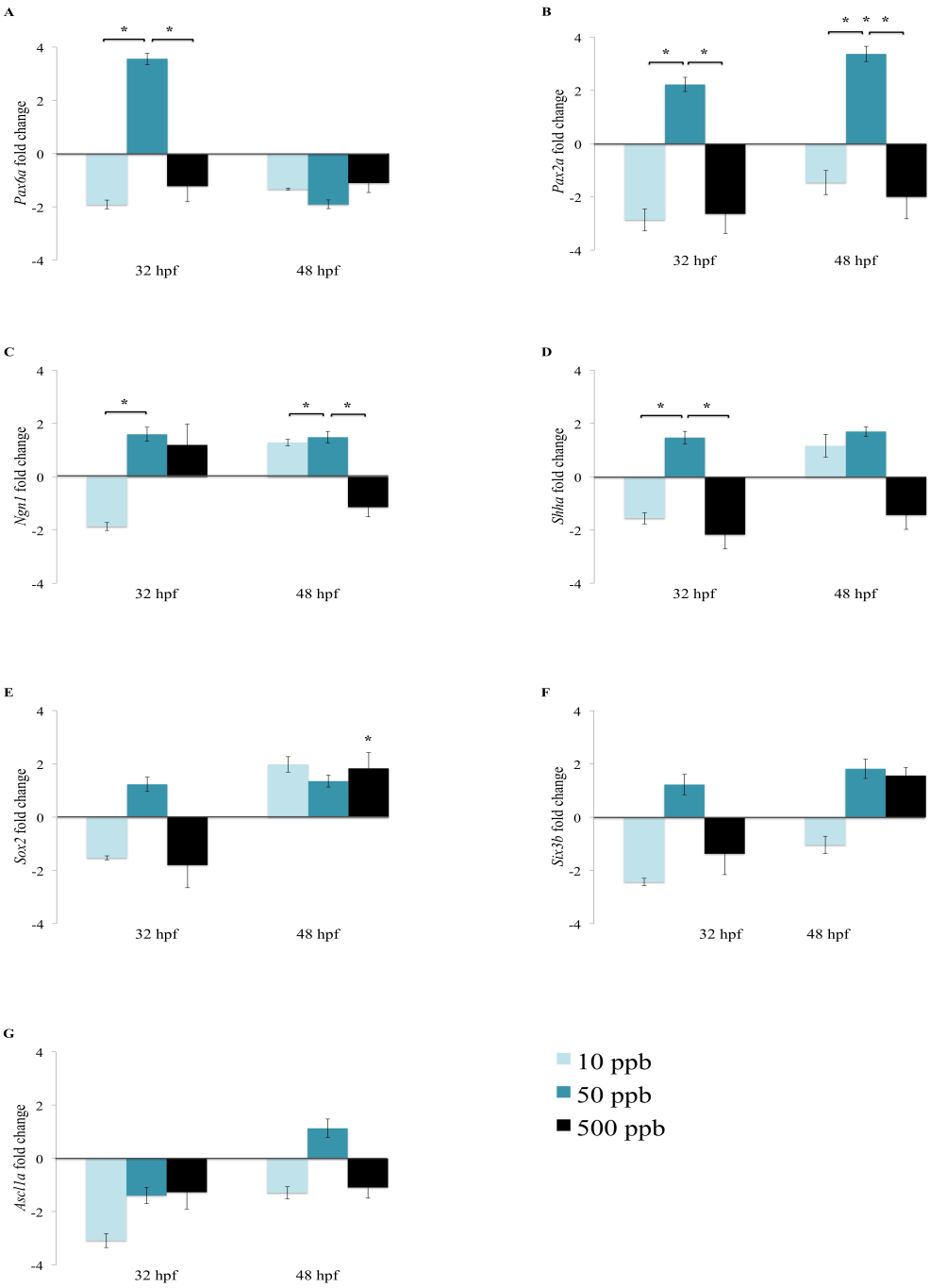

Figure 2.5: RT-qPCR results of gene expression associated with eye development. RT-qPCR results of gene expression associated with eye development (A-G) and neurogenesis following 10, 50, and 500 ppb $AsNaO_2$ treatment conditions from ~1 hpf until time of extraction at 32 and 48 hpf. Bars represent fold change (Pfaffl method) relative to control (0 ppb) ± SEM. $\overset{*}{\text{—}}$ indicates significant difference between two treatment groups, $\overset{*}{\uparrow}$ indicates significant difference between treatment and control $p \leq 0.05$, see Appendix Table A4 for specific p values.

CHAPTER 3

DISCUSSION

The findings of the present study indicate that arsenic exposure may affect eye development and retinogenesis in the zebrafish. Analysis of expression of genes key to retinal and RPE development coupled with the observed thinning of the RPE and an increase in eye size over 14 days under arsenic treatment conditions suggest that arsenic exposure may affect eye development and retinogenesis. This can be seen in an overall larger eye diameter at 14 dpf under treatment conditions as well as a decrease in both the posterior and peripheral RPE. The RPE is a pigmented layer that acts as an interface between the neural retina and the choroid and sclera, absorbing stray light that has passed through the photoreceptors (Sparrow et al. 2010). Besides protecting the integrity of visual images from additional light it also acts as a blood-retina barrier and contains receptors necessary for retina – sclera communication. The sclera, primarily composed of collagens and fibers, is responsible for maintaining the shape of the eye while providing protection for intraocular structures and acting as a stable base for ciliary muscle contractions.

Normal scleral development is highly dependent on cues from the neural retina and RPE. These include dopamine and growth factors FGF-β and TGF-β (Zhang and Wildsoet, 2015). Dopamine antagonist apomorphine has been shown to inhibit scleral growth and dopamine combined with FGF-β has been seen to reduce scleral thinning (Rohrer et al. 1995). FGF-β is produced in the developing neural retina (Desire, 1998). The RPE contains FGFR1, FGFR2 (Rosenthal, 2005), and Dopamine receptors to initiate signals to the sclera for growth. The RPE also has the ability to produce FGF-β and FGF-β, TGF-β specifically have been shown to inhibit scleral thinning by decreasing collagen degradation (Uchida et al. 2008), and may modulate

scleral growth via cell matrix interactions (Shelton et al. 2008). Arsenic has also been seen to have an effect on DAR mRNA expression levels (Rodríguez et al. 2010) and TGF-β production (Allison et al. 2013) which may also be playing a role in reduced communication of growth factor signals to the sclera.

The arsenic-induced decrease in RPE thickness over time could impact the ability of the RPE to communicate with the sclera. Miscommunication of the RPE with important growth factors such as FGF-β and TGF-β could result in collagen degradation and thinning of the sclera. As the sclera thins, it loses its strength and elasticity, which leads to abnormal eye growth and the potential for an increased vitreous chamber at later stages. A thinning of the sclera has been associated with the development of myopia, or nearsightedness, and is characterized by an increase in axial length of the eye causing refractive error (McBrien and Gentle, 2003). Axial length is most commonly elongated when there is scleral thinning and an increase in the vitreous chamber.

The length of the retina, from the ganglion cell layer to the RPE, was also measured axially (see Appendix Figure A3) but the data was not significant. Other cellular layers (such as ganglion, inner plexiform, and nuclear cell layers) under treatments remained similar to control values. The thinning of the RPE and possible scleral degradation could account for the increase seen in overall all eye size under treatment conditions even though the retina does not appear to be increasing at the same rate axially. There may be extraneous fluid stored in the eye that is not visible by histological analysis, or that may have been lost during the fixation process.

To investigate the effects of arsenic on a molecular level, we studied genes essential for successful eye formation within 60 hpf. The paired box genes, *Pax6a* and *Pax2a,* are known to be involved in retinogenesis, and co-expression is necessary for the development of the RPE.

Both Pax6a and Pax2a bind to the RPE genetic marker *Mitf*. Mitf or micropthalmia-associated transcription factor is involved in early development of the eye and null mutants result in transdifferentiation of the RPE into the neural retina. Specifically, *Mitf* expression patterning in the optic vesicle is necessary to mark boundaries of the presumptive RPE and distinguish it from the neural retina (Baumer et al. 2003). At 500 ppb $AsNaO_2$ treatments, both *Pax6a* and *Pax2a* are downregulated at 48 hpf relative to controls (Figure A5).

Shha, *Six3b*, and *Sox2* are also involved in the development of the RPE. Differential expression of these key developmental genes supports histological data of decreasing RPE thickness with higher exposure levels of arsenic. Although differences in *Six3b* gene expression were not significant, *Six3b* was upregulated at 48 hpf. In a study done on early embryogenesis in *Xenopus*, an overexpression of *Six6 (Six3b)* led to a reduction in RPE cells (Bernier et al. 2000). Therefore, arsenic effects on early *Six3b* expression may be playing a role in the reduced zebrafish RPE cellular layer seen at 14 dpf. Similarly, continued expression of *Sox2*, which has been studied in avian RPE development, disrupts RPE morphogenesis and cellular differentiation (Yasou et al. 2009).

At 48 hpf there is an upregulation of *Sox2* in all three treatments. *Pax6a*, Pax*2a*, *Six3b*, and *Sox2* gene expression levels that correlate with disrupted RPE development are consistent with our own gene expression observations at 48 hpf under 500 ppb treatment (Figure A5). This may suggest a mechanism for RPE is thinning at later stages (Figure 3.2).

A. Normal Developing Eye

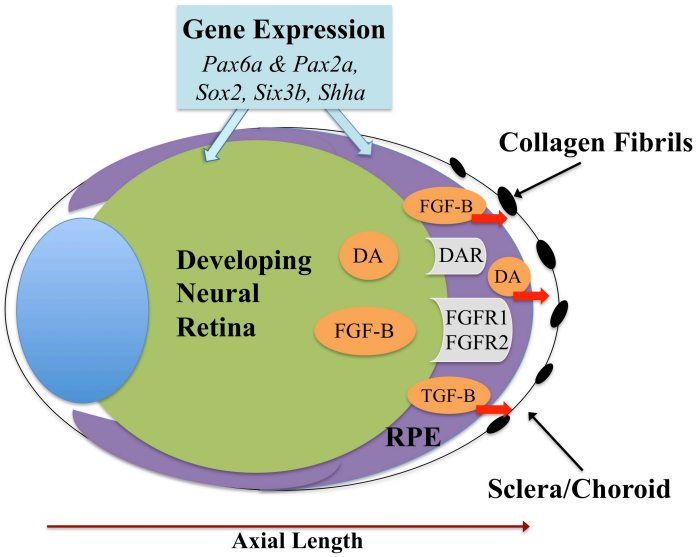

Figure 3.1: Predicted molecular interactions in the normal developing eye. *Pax6a, Pax2a*, *Sox2*, *Six3b*, and *Shha* are expressed in the developing neural retina and RPE. Growth factors dopamine (DA) and FGF-β are produced in the neural retina, pass through the RPE via dopamine and FGF receptors and ultimately are transported to the sclera. FGF-β and TGF-β are also produced by the RPE and are transported to the sclera. These growth factors stimulate collagen fibril formation and prevent collagen degradation. Both are necessary for the development of a functional sclera.

B. Developing Eye Under Arsenic Treatment conditions

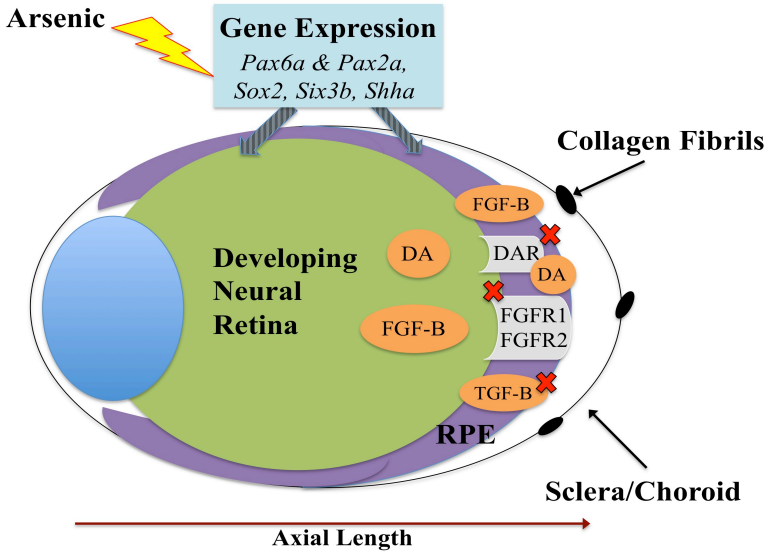

Figure 3.2: Predicted molecular interactions in the developing eye following arsenic exposure. *Pax6a*, *Pax2a*, *Sox2*, *Six3b*, and *Shha* are differentially expressed relative to controls in the developing neural retina and RPE. The effect of arsenic on expression of these genes may be inhibiting proper development of the RPE (observed by RPE thinning), resulting in an inability of important growth factors from the neural retina to communicate with the sclera or the inability of the RPE to properly produce FGF-β and TGF-β. Without functional communication of growth factors to the sclera collagen fibrils begin to degrade and the sclera loses its stability. This could result in an overall larger eye and an increased chance for the development of myopia.

$AsNaO_2$ treatments of 10 and 500 ppb show a similar downregulation of *Pax6a*, *Pax2a*, and *Ascl1a* at both 32 and 48 hpf. Given the importance of *Pax6a* / *Pax2a* interactions as transcription factors in the developing retina and RPE this may explain the similarities of increased eye size at 14 dpf. Although there is a significant increase in eye size at 14 dpf under all treatments horizontally and 10 and 500 ppb vertically there is not a noticeable decrease in RPE cell layer thickness at 10 and 50 ppb. Given the ability of the zebrafish to regenerate retinal cells, it is possible that we would not observe a difference in 10 and 50 ppb treatments, but communication between the sclera and neural retina through the RPE may still be compromised.

Since there is a distinct thinning of the RPE at 14 dpf under 500 ppb treatments this may be indicative of higher arsenic concentrations inhibiting or delaying their regenerative capacity.

To our knowledge, this study is the first to link arsenic to the developing eye and its indirect impact on RPE and scleral development through changes in genes critical to the developing visual system. The molecular analysis of core genes related to retina and RPE development coupled with the histological thinning of the RPE and an increase in eye size over 14 days under treatment conditions suggest arsenic may affect eye development and retinogenesis. The thinning of the RPE cellular layer suggests disruption in RPE development. If the RPE is not properly formed its role in the communication of growth signals from the retina to the sclera may be compromised. Improper sclera development could lead to decreased elasticity and collagen degradation, allowing the vitreous chamber and overall eye to increase in size. In this study the fish have not yet reached an age at which their vitreous chambers are developed. It would be interesting to further investigate morphology and histology in adult zebrafish who were exposed to arsenic during embryonic development to see if the RPE continues to thin under treatment and if it is coupled with increased vitreous chambers.

CHAPTER 4

FUTURE DIRECTIONS

Arsenic exposure produced larger eyes and a thinned RPE layer in zebrafish larvae as well as differential expression of genes key to retinogenesis. In order to gain a clearer understanding of how arsenic may be affecting eye development, a variety of analyses using the zebrafish model can be explored. These include studying arsenic impact on zebrafish eye regeneration, protein expression analysis, and behavioral studies to test vision impairment.

4.1 Exploring the Impacts of Arsenic on Zebrafish Regeneration

An inhibition or delay in the zebrafish regenerative capacity could be explored further by an embryonic exposure to arsenic followed by morphological and histological analysis of adult fish. Zebrafish are known to regenerate eyes after mechanical and light injury (Thummel et al. 2010). If fish were treated with arsenic during embryogenesis and later sustained damage to the eye as adults, it would be interesting to see if their regenerative response was still at full capacity or hindered by arsenic exposure.

Although in this study, *Ascl1a* did not show a significant difference in expression from control levels, it may be more informative to examine *Ascl1a* expression in the adult. *Ascl1a* is considered to be the master gene in zebrafish eye regeneration, being one of the first genes to be upregulated upon injury activating WNT, Notch, and Hedgehog pathways in the regeneration response (Ramachandran et al. 2010; 2011). If *Ascl1a* is not upregulated upon injury then other factors could be inhibited in the regenerative pathway including, but not limited to, activation of Mueller glia for phagocytosis, dedifferentiation and proliferation of retinal progenitor cells, and differentiation of cells by genetic markers.

4.2 Protein Analysis

It would also be interesting to explore how arsenic exposure may be affecting protein expression of the target genes. Protein expression can be analyzed by western blot and protein-arsenic interactions can be explored via an arsenic pull down assay. Western blot analysis would give insight on the effects of arsenic on successful protein translation. If arsenic exposure resulted in decreased protein expression, then protein formation may have been inhibited or post-translational modifications may have been disrupted. Pax6 is known to be phosphorylated by homeodomain-interacting protein kinase 2 (HIPK2; Kim et al. 2006), thus phosphorylation could be investigated using phospho-specific antibodies. An arsenic-biotin pull down assay would provide evidence that arsenic was interacting directly with target proteins (Heredia-Moya and Kirk 2008, Zhang et al. 2015). AsIII has a high binding affinity to sulfyhydryl and cysteine groups in polypeptides (Shengwen et al. 2013). Both Pax6 and Pax2 contain cysteine groups. There are 7 total cysteines in Pax6, 4 of which are in the paired domain, and 4 total cysteines in Pax2 (3 in paired domain). An interaction of arsenic with a Pax6 cysteine could result in a conformational change and could potentially hinder the ability of Pax6 to bind and regulate gene expression necessary for eye development.

4.3 Behavioral Assays

Given that our data suggest that arsenic affects eye development both morphologically and genetically, it may also be inhibiting visual function. Improper eye formation or an inability to transmit visual signals from the eye to the brain could impair vision. Behavioral assays could identify the functional impact of the observed genetic and morphological changes. These assays could include an optokinetic response (OKR), which is used to track eye movement in response to a stimulus (Brockerhoff, 2006). Other visual behavioral tests include the startle response

(SR), optomotor response (OR), and escape response (ER) (Chhetri et al. 2014). Although a behavioral assay is not yet available to specifically measure myopia in zebrafish, there have been studies done using laser technology (spectral domain-optical coherence tomography) to measure axial length of the retina in the developing zebrafish (Collery et al. 2014). This method is non-invasive, allowing the ability to track axial length throughout development, and provides a more acute insight into myopia progression in the zebrafish model.

The zebrafish offers a variety of ways to study toxicological impacts of arsenic on neurogenesis and the developing eye. By utilizing the above technologies we can gain greater insights as to the direct and indirect impacts of arsenic exposure on proteins, visual functionality, and the regenerative capacity of the zebrafish. Given that eye development is an essential part of neurogenesis, knowledge gained from these studies could be related to arsenic effects on early child development and be implemented to show the severity of arsenic on neurogenesis.

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APPENDIX
SUPPLEMENTAL MATERIAL FOR A GREATER UNDERSTANDING OF FINDINGS FROM THE CURRENT STUDY

Table A1: BioRad thermocycler protocol. BioRad thermocycler protocol used for RT-qPCR analysis.

Thermocycler protocol					
Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
95°C for 30 sec	95°C for 5 sec	Annealing temp for 45 sec	Repeat steps 2 – 3 39x	95°C for 10 sec	Melt curve analysis

Table A2: qPCR specifications. List of genes, reverse and forward primers, annealing temperature, primer nm used, and % efficiency (E) for all genes analyzed by RT-qPCR.

Gene	F primer 5'→ 3'	R primer 5'→ 3'	Annealing Temperature C°	Reaction Quantity (nm)	% E
Pax6a	CTGACGTTTTTGCACGAGAA	AAAGGATACTGGCGTTGTGG	50.4	300	106
Pax2a	TCTCACCCGCAGTACACAAC	CTAGTGGCGGTCATAGGCAG	58.5	300	111
Ngn1	CAGAAGCAGGGCAAGTCAAG	CACTACGTCGGTTTGCAAGT	51.6	350	102
Six3b	CACATCTTTTCCTGCCCAAT	GATGGACTCGTGCTTGTTGA	53.5	400	105
Sox2	GAGTCTAGTTCGAGTCCGCC	CAGGTGCGCTCTGGTAATGT	59.8	400	103
Shha	TGTCCTCGACAACTCAACGG	TCCGTGTATATCCGCTGCAC	57.7	300	94
Ascl1a	GCCAGACGGAACGAGAGAGA	AGGGTTGCAAAGCCGTTG	60.7	400	105
Bactin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	58.3	400	102

Table A3: P values of morphological and histological data. A one-way ANOVA was performed to test for significance between treatments at 4, 7, and 14 hpf. Significance was confirmed using post-hoc Tukey's test. Upon failure of Levene's test, Games and Howell post-hoc was used to determine significance among treatments (corresponds to Figures 2 and 4).

Measured	Treatments Compared (µg/L)	P value
Morphology at 4 dpf		
Horizontal eye diameter	0-10	0.903
	0-50	0.91
	0-500	0.12
	10-50	1
	10 - 500	0.072
	50 – 500	0.073
Vertical eye diameter	0-10	0.971
	0-50	0.84
	0-500	0.616
	10-50	0.98
	10-500	0.86
	50-500	0.977
Morphology at 7 dpf		
Horizontal eye diameter	0-10	0.899
	0-50	0.998
	0-500	0.997
	10-50	0.815
	10 - 500	0.803
	50 – 500	1
Vertical eye diameter	0-10	0.789
	0-50	0.774
	0-500	0.811
	10-50	1
	10-500	1
	50-500	1
Morphology at 14 dpf		
Horizontal eye diameter	0-10	0.001
	0-50	0
	0-500	0.011
	10-50	0.175

Measured	Treatments Compared (µg/L)	P value
Histology at 4 dpf		
Posterior RPE	0-10	0.789
	0-50	1
	0-500	0.662
	10-50	0.954
	10 - 500	0.702
	50 – 500	0.968
Peripheral RPE	0-10	0.936
	0-50	0.694
	0-500	0.293
	10-50	0.157
	10-500	0.888
	50-500	0.694
Histology at 7 dpf		
Posterior RPE	0-10	0.981
	0-50	0.993
	0-500	0.865
	10-50	0.949
	10 - 500	1
	50 – 500	0.949
Peripheral RPE	0-10	0.999
	0-50	0.995
	0-500	0.803
	10-50	0.999
	10-500	0.736
	50-500	0.664
Histology at 14 dpf		
Posterior RPE	0-10	0.889
	0-50	0.112
	0-500	0.892
	10-50	0.037

Table A3 continued

	10 - 500	0.678
	50 – 500	0.871
Vertical eye diameter	0-10	0.025
	0-50	0.115
	0-500	0.028
	10-50	0.266
	10-500	0.939
	50-500	0.461

	10 - 500	0.311
	50 – 500	0.311
Peripheral RPE	0-10	1
	0-50	0.817
	0-500	0.336
	10-50	0.066
	10-500	0.789
	50-500	1

Table A4: P values of RT-qPCR data. A one-way ANOVA was performed to test for significance between treatments at 32 and 48 hpf. Significance was confirmed using post-hoc Tukey's test. Upon failure of Levene's test, Games and Howell post-hoc was used to determine significance among treatments (corresponds to Figure 2).

32 hpf		
Gene	Treatments Compared (µg/L)	P value
Pax6a	0-10	0.674
	0-50	0.062
	0-500	0.936
	10-50	0.005
	10 - 500	0.945
	50 – 500	0.018
Pax2a	0-10	0.984
	0-50	0.059
	0-500	0.988
	10-50	0.021
	10-500	1
	50-500	0.022
Ngn1	0-10	0.816
	0-50	0.66
	0-500	0.899
	10-50	0.011
	10 - 500	0.48
	50 – 500	0.999
Six3b	0-10	0.582
	0-50	1
	0-500	0.961

48 hpf		
Gene	Treatments Compared (µg/L)	P value
Pax6a	0-10	0.666
	0-50	0.106
	0-500	0.85
	10-50	0.651
	10 - 500	0.988
	50 – 500	0.45
Pax2a	0-10	0.998
	0-50	0.05
	0-500	0.831
	10-50	0.041
	10-500	0.857
	50-500	0.03
Ngn1	0-10	0.999
	0-50	0.041
	0-500	0.996
	10-50	0.042
	10 - 500	0.987
	50 – 500	0.063
Six3b	0-10	0.586
	0-50	0.155
	0-500	0.335

Table A4 continued

	10-50	0.051		10-50	0.761
	10-500	0.65		10-500	0.964
	50-500	0.899		50-500	0.956
Sox2	0-10	0.998	*Sox2*	0-10	0.325
	0-50	0.992		0-50	0.233
	0-500	0.999		0-500	0.012
	10-50	0.973		10-50	0.277
	10 - 500	0.987		10 - 500	1
	50 – 500	0.973		50 – 500	0.816
Shha	0-10	1	*Shha*	0-10	0.731
	0-50	0.341		0-50	0.699
	0-500	0.903		0-500	0.795
	10-50	0.022		10-50	1
	10-500	0.681		10-500	0.238
	50-500	0.039		50-500	0.218
Ascl1a	0-10	0.654	*Ascl1a*	0-10	0.935
	0-50	0.854		0-50	0.998
	0-500	0.933		0-500	0.998
	10-50	0.505		10-50	0.876
	10 - 500	0.6		10 - 500	0.994
	50 – 500	0.977		50 – 500	0.961

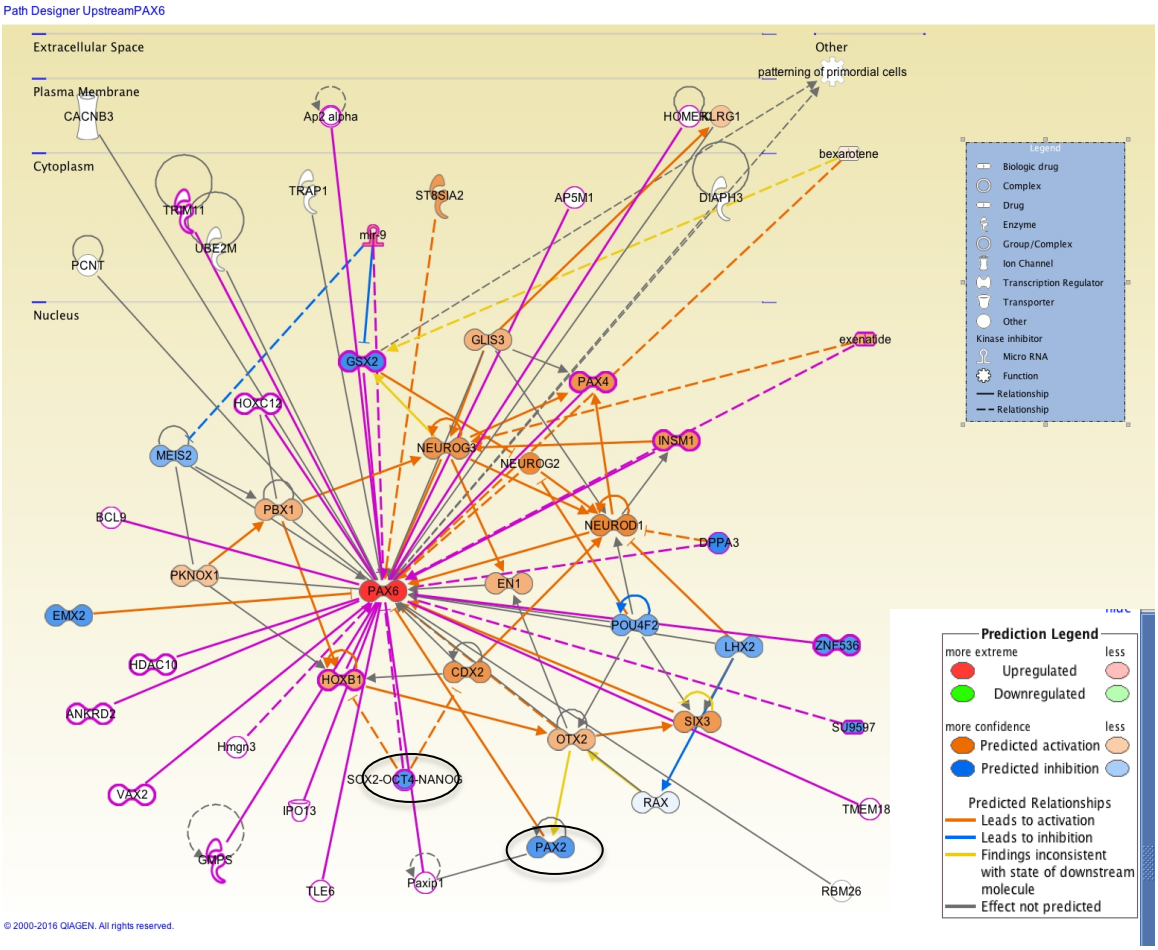

Figure A1: Genetic pathway designed by Ingenuity software displaying genes that are upstream of *Pax6*. Genes circled in black were chosen as target genes for this study.

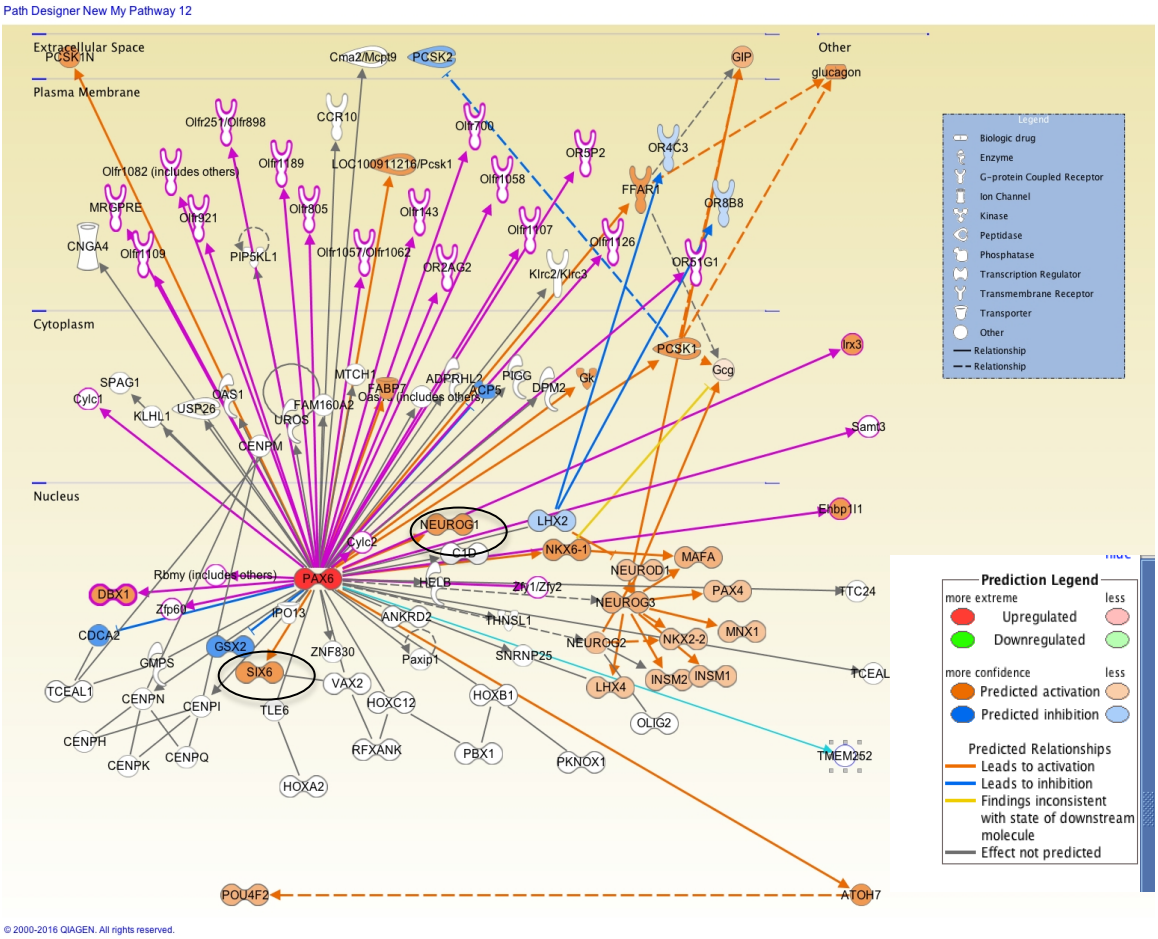

Figure A2: Genetic pathway designed by Ingenuity software, displaying genes that are downstream of *Pax6*. Genes circled in black were chosen as target genes for this study.

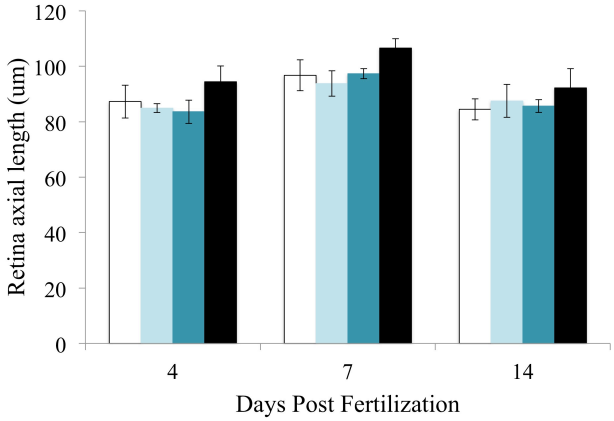

Figure A3: Retinal axial length. Retinal axial length measured from the ganglion cell layer to the RPE cell under 0, 10, 50, and 500 ppb AsNaO2 treatments from 1 – 72 hpf at 4, 7, and 14 dpf. Bars represent cell layer thickness measured in µm ± SEM.

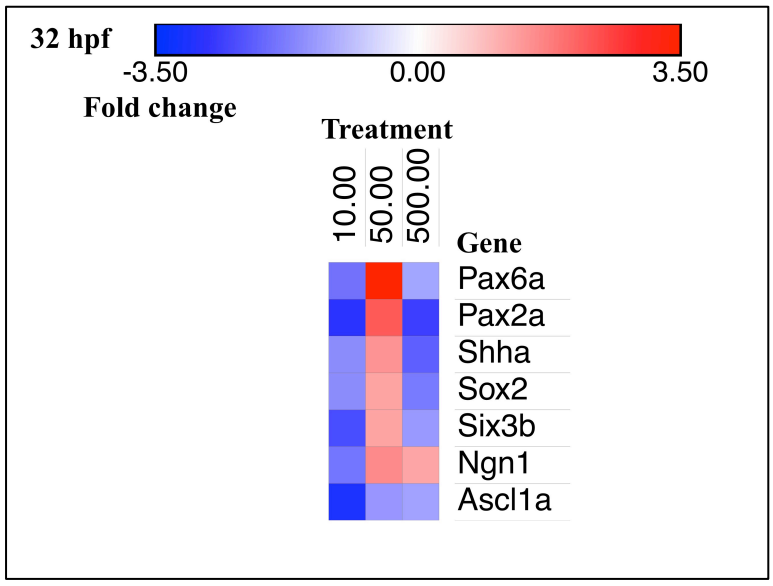

Figure A4: Heat map of target gene fold change relative to control at 32 hpf. Heat map of target gene fold change (Pfaffl method) relative to control at 32 hpf under 10, 50, and 500 ppb AsNaO2 treatments from 1 – 72 hpf. Genes are clustered by similarities among fold change. Colors represent a gene that is downregulated (blue) or upregulated (red) compared to control under treatments. This heat map was designed by Morpheus® software.

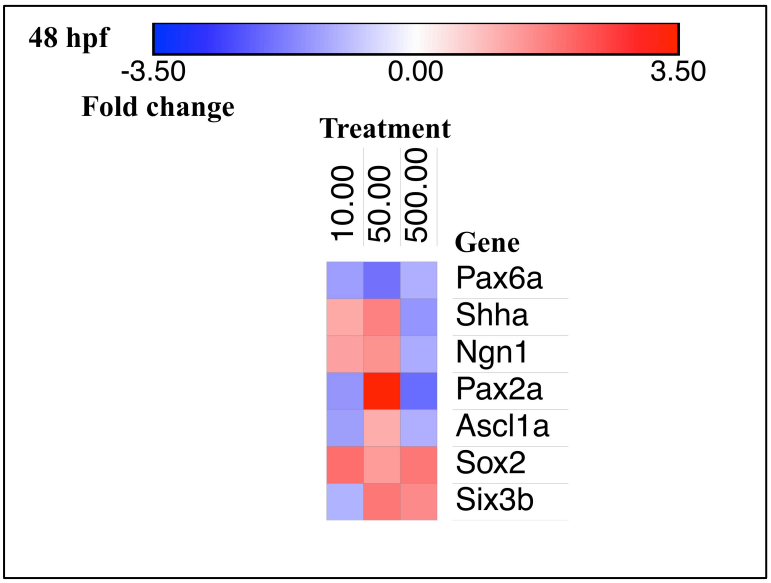

Figure A5: Heat map of target gene fold change relative to control at 48 hpf. Heat map of target gene fold change (Pfaffl method) relative to control at 48 hpf under 10, 50, and 500 ppb AsNaO2 treatments from 1 – 72 hpf. Genes are clustered by similarities among fold change. Colors represent a gene that is downregulated (blue) or upregulated (red) compared to control under treatments. This heat map was designed by Morpheus® software.

BIOGRAPHY OF THE AUTHOR

Remy Babich was born in Pittsburgh, Pennsylvania on December 4th 1992. Remy and her family shortly thereafter moved to Harrisburg, Pennsylvania where she lived her childhood. She graduated from Central Dauphin East High in May 2011. During this time Remy also pursued Cosmetology, and received her Pennsylvania Cosmetologists license in 2012. Upon graduation, Remy attended Penn State University Harrisburg campus for 2 years and continued her career as a beautician. She was then transferred to Penn State University Park where she finished her B.S. degree in Biology. Remy then continued to the University of Maine to pursueher Master's Degree in Biochemistry in the Department of Molecular and Biomedical Sciences. Remy is a candidate for the Master of Science degree in Biochemistry from the University of Maine in August 2018.