The Effects of Embryonic Arsenic Exposure on Muscle and Olfactory Development in Killifish (Fundulus heteroclitus)

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THE EFFECTS OF EMBRYONIC ARSENIC EXPOSURE ON MUSCLE AND OLFATORY DEVELOPMENT IN KILLIFISH (*FUNDULUS HETEROCLOTUS*).

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

by
Dana Bethany Szymkowicz
December 2018

Accepted by:
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Dr. Susan Chapman
ARSENIC is an environmental contaminant that is commonly found in drinking water, rice, and other food sources around the world. Many epidemiological studies have reported that *in utero* exposures to arsenic can significantly reduce muscle growth and cause neurodevelopmental abnormalities such as reduced IQ, impaired memory and spatial learning, and sensory functions. However, the mechanisms behind such effects are not well known. Because of this, we wanted to explore the potential mechanisms by which embryonic exposure to environmentally-relevant levels of arsenic are not only impairing growth but also inducing behavioral changes due to altered sensory neuron function. We were also interested in knowing whether these changes are permanent and remain after the exposure is removed.

Killifish (*Fundulus heteroclitus*) are a commonly used model organism for developmental studies, as they can produce a large number of transparent eggs which allows for greater statistical power. They are readily cultured in the laboratory, so we are also able observe changes over the lifetime of the organisms. Further, previous studies have shown that environmentally-relevant levels of arsenic produce adverse effects in killifish similar to those that cause deficits in humans. Other models, such as zebrafish or rodents, require much higher levels of arsenic to produce similar effects. Therefore, we exposed killifish embryos to 0, 50, 200 or 800 parts per billion (ppb) sodium arsenite. After hatching, the juvenile fish were removed from the exposures and raised in clean water until 52 weeks of age. A second study was conducted to determine the effects of arsenic on olfactory development and function, where embryos were exposed to either 0, 10, 50 or 200 ppb arsenite during embryogenesis and raised in clean water until 40 weeks of age. These studies were designed to examine the effects of exposures during critical points of development (embryonic/fetal) and to investigate if deficits persisted well after the exposure was completed.
The results of the first arsenic exposure revealed that at 16 weeks post-exposure, condition factors (CF; weight/length$^3$) were significantly reduced by 12-18% in 200 and 800ppb fish compared to controls. At 28 weeks, all three exposure groups had CFs significantly lower than controls. By 52 weeks, however, CFs were similar in all groups. To examine mechanisms responsible for the growth lag in exposed fish, both trunk skeletal muscle fiber density and skeletal muscle expression of insulin-like growth factor-1 (IGF-1) and its receptor, IGF1-R, were examined. Although there were no significant reductions in muscle fiber density at any time point, both IGF-1 and IGF1-R levels were significantly upregulated in exposed fish at 16 weeks of age (2-2.8-fold greater), and these differences persisted through 52 weeks. These results indicate that reductions in growth may be compensated for by upregulation of the IGF growth pathway, and that embryonic arsenic exposure does not permanently impair growth. However, the consequences of constant IGF upregulation over the lifespan of the organism are unknown.

Previous studies have shown that in vitro exposure to arsenic can reduce the expression of transcription factors needed for myogenesis, thus inhibiting their differentiation from stem cells. In order to determine if arsenic targets muscle satellite cells as in vivo, we used fish from the previous study to conduct an induced injury experiment. At 28, 40 and 52 weeks of age, three killifish from each exposure replicate were anesthetized, injected with cardiotoxin below the dorsal fin, and allowed to recover until 3, 7, or 10 days post-injury. Baseline PCNA (a marker of proliferation) and collagen levels were also assessed prior to injury. Immunohistochemical analysis revealed consistent increases in both proliferative cells and collagen expression in exposed fish at all time points in uninjured animals. During the repair process, PCNA expression was reduced in exposed fish, and instead, increases in collagen expression were seen. These results indicate that an embryonic-only arsenic exposure impaired proliferation and differentiation of muscle satellite cells and resulted in the enhancement of a fibrotic resolution to injury.
Finally, we conducted a second embryonic exposure with slightly lower arsenic levels (0, 10, 50, or 200 ppb arsenic) to assess the effects of embryonic-only arsenic exposure on neurogenesis, using the olfactory epithelium and olfactory sensory neurons (OSNs) as models. To test the function of crypt, ciliated, and microvillus OSNs within the epithelium, we performed odorant response tests at 0, 2, 4, 8, 16, 28, and 40 weeks of age using female ovarian extracts, taurocholic acid (TCA), and an amino acid mixture as odorants for each cell type, respectively. Our results showed that exposed killifish took significantly longer to respond to the ovarian extracts and TCA odorants through week 28, and the number or percentage of fish per tank responding to each odorant was also significantly lower in exposed groups compared to controls. Killifish responses to amino acids were not significantly impaired by arsenic exposure. Immunohistochemistry was used to determine if the number of proliferating cells (PCNA), neural stem cells (Sox2), ciliated cells (calretinin), and microvillus cells (Gαi3) in the olfactory epithelium were reduced due to embryonic arsenic exposure. The results of this study show no changes in numbers of PCNA positive cells until week 16, in which their numbers are significantly greater in exposed fish compared to controls. Sox2 expression is decreased through week 16 and calretinin expression (ciliated OSNs) is consistently lower in exposed groups from week 4 to week 28. These changes in proliferation and differentiation of stem cells in the olfactory epithelium are very similar to those seen in muscle satellite cells after embryonic-only exposure, and these results indicate that arsenic impairs the function of stem cells in both neural and muscle tissue, and these deficits continue throughout adulthood.
Overall, it appears that arsenic has long-term effects on the presence and function of both muscle and neural stem cells. In spite of attempts to compensate for these deficits, such as upregulating IGF-1 levels in skeletal muscle and increasing proliferation in skeletal muscle and the olfactory epithelium, it is clear that losses in muscle growth are somewhat ameliorated into adulthood while olfactory epithelial functions are not fully recoverable once the exposure has been removed.
DEDICATION

This dissertation is dedicated to my father, Patrick Szymkowicz, for instilling in me a passion for quality science and exploring the unknown, and to my mother, Christine Szymkowicz, for never letting me forget who I was and believing in me when no one else did.
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Arsenic in the environment</td>
<td>1</td>
</tr>
<tr>
<td>Human health effects of arsenic exposure</td>
<td>3</td>
</tr>
<tr>
<td>Arsenic as a developmental toxicant</td>
<td>6</td>
</tr>
<tr>
<td>Killifish (<em>Fundulus heteroclitus</em>) as a model organism</td>
<td>9</td>
</tr>
<tr>
<td>Muscle development in mammals</td>
<td>10</td>
</tr>
<tr>
<td>Muscle development in fish</td>
<td>14</td>
</tr>
<tr>
<td>Olfactory development in mammals</td>
<td>17</td>
</tr>
<tr>
<td>Olfactory development in fish</td>
<td>20</td>
</tr>
<tr>
<td>Dissertation goals and objectives</td>
<td>24</td>
</tr>
<tr>
<td>References</td>
<td>26</td>
</tr>
<tr>
<td>II. EMBRYONIC-ONLY ARSENIC EXPOSURE IN KILLIFISH (<em>FUNDULUS HETEROCLITUS</em>) REDUCES GROWTH AND ALTERS MUSCLE IGF LEVELS ONE YEAR LATER</td>
<td>35</td>
</tr>
<tr>
<td>Abstract</td>
<td>36</td>
</tr>
<tr>
<td>Introduction</td>
<td>37</td>
</tr>
<tr>
<td>Methods</td>
<td>40</td>
</tr>
<tr>
<td>Results</td>
<td>44</td>
</tr>
<tr>
<td>Discussion</td>
<td>48</td>
</tr>
<tr>
<td>Conclusions</td>
<td>52</td>
</tr>
</tbody>
</table>
Table of Contents (continued)

| References | 54 |
| Figures    | 58 |

III. EMBRYONIC-ONLY ARSENIC EXPOSURE ALTERS SKELETAL MUSCLE SATELLITE CELL FUNCTION IN KILLIFIISH (*FUNDULUS HETEROCLETUS*)

| Abstract       | 71 |
| Introduction   | 72 |
| Methods        | 75 |
| Results        | 79 |
| Discussion     | 84 |
| Conclusions    | 89 |
| References     | 90 |
| Figures        | 93 |

IV. OLFATORY STEM CELLS AND ASSOCIATED BEHAVIORS ARE REPRESSED LONG-TERM DUE TO EMBRYONIC ARSENIC EXPOSURES IN KILLIFIISH (*FUNDULUS HETEROCLETUS*)

| Abstract       | 116 |
| Introduction   | 118 |
| Methods        | 121 |
| Results        | 125 |
| Discussion     | 130 |
| Conclusions    | 136 |
| References     | 137 |
| Figures        | 141 |

V. CONCLUSION

| References     | 156 |
| References     | 165 |
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Hatching success and fish survival of killifish is not altered after</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>embryonic-only arsenic exposure.</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Embryonic-only arsenic reduces condition factors of killifish until 40 weeks</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>of age.</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Embryonic-only arsenic causes an increase in percent occupancy in the</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>bottom of the tank.</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>Representative images of killifish trunk muscle cross sections</td>
<td>63</td>
</tr>
<tr>
<td>2.5</td>
<td>Arsenic increases muscle fiber density at 16 weeks of age.</td>
<td>64</td>
</tr>
<tr>
<td>2.6</td>
<td>Embryonic arsenic exposure increases skeletal muscle IGF-1R mRNA levels.</td>
<td>66</td>
</tr>
<tr>
<td>2.7</td>
<td>Embryonic arsenic exposure increases skeletal muscle IGF-1 mRNA levels.</td>
<td>68</td>
</tr>
<tr>
<td>3.1</td>
<td>Baseline collagen expression in muscle is increased following embryonic</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>arsenic exposure.</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Representative images of collagen expression in 28 week old killifish muscle</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>after cardiotoxin injury.</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Embryonic-only arsenic exposure increases collagen expression after injury.</td>
<td>96</td>
</tr>
<tr>
<td>3.4</td>
<td>Immunoblots of killifish PCNA and myogenin.</td>
<td>97</td>
</tr>
<tr>
<td>3.5</td>
<td>Baseline PCNA expression in killifish trunk muscle is increased after</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>embryonic arsenic exposure.</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>PCNA expression is reduced in skeletal muscle following a cardiotoxin injury</td>
<td>100</td>
</tr>
</tbody>
</table>
List of Figures (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7</td>
<td>Embryonic-only arsenic exposure reduces PCNA expression up to 10 days post-injury</td>
</tr>
<tr>
<td>3.8</td>
<td>Correlations between PCNA and collagen</td>
</tr>
<tr>
<td>3.9</td>
<td>Correlations between PCNA and myogenin</td>
</tr>
<tr>
<td>S3.1</td>
<td>Representative images of collagen expression in killifish trunk muscle after cardiotoxin injury at 40 weeks</td>
</tr>
<tr>
<td>S3.2</td>
<td>Representative images of collagen expression in killifish trunk muscle after cardiotoxin injury at 52 weeks</td>
</tr>
<tr>
<td>S3.3</td>
<td>PCNA expression in killifish trunk muscle after cardiotoxin injury at 40 weeks</td>
</tr>
<tr>
<td>S3.4</td>
<td>PCNA expression in killifish trunk muscle after cardiotoxin injury at 52 weeks</td>
</tr>
<tr>
<td>S3.5</td>
<td>Baseline myogenin expression in killifish trunk muscle</td>
</tr>
<tr>
<td>S3.6</td>
<td>Representative images of myogenin expression in killifish trunk muscle after cardiotoxin injury at 28 weeks</td>
</tr>
<tr>
<td>S3.7</td>
<td>Representative images of myogenin expression in killifish trunk muscle after cardiotoxin injury at 40 weeks</td>
</tr>
<tr>
<td>S3.8</td>
<td>Representative images of myogenin expression in killifish trunk muscle after cardiotoxin injury at 28 weeks</td>
</tr>
</tbody>
</table>
List of Figures (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3.9</td>
<td>Embryonic-only arsenic exposure does not alter myogenin expression 10 days post-injury</td>
</tr>
<tr>
<td>S3.10</td>
<td>Correlations between collagen and myogenin</td>
</tr>
<tr>
<td>4.1</td>
<td>Embryonic arsenic exposure increases start time and reduces killifish response to pheromones</td>
</tr>
<tr>
<td>4.2</td>
<td>Embryonic arsenic exposure increases start time and reduces killifish response to TCA through 40 weeks of age</td>
</tr>
<tr>
<td>4.3</td>
<td>Embryonic arsenic exposure increases start time and reduces killifish response to amino acids through 40 weeks of age</td>
</tr>
<tr>
<td>4.4</td>
<td>Representative confocal images of week 0 immunohistochemistry of olfactory epithelia</td>
</tr>
<tr>
<td>4.5</td>
<td>Representative confocal images of week 28 immunohistochemistry of olfactory epithelia</td>
</tr>
<tr>
<td>4.6</td>
<td>Embryonic arsenic exposure increases PCNA expression and reduces Sox2 expression in the olfactory epithelium through week 28</td>
</tr>
<tr>
<td>4.7</td>
<td>Embryonic arsenic exposure decreases calretinin and $G_{\alpha i3}$ expression in the olfactory epithelium through week 16</td>
</tr>
<tr>
<td>4.8</td>
<td>Correlations between PCNA/Sox2 and percent response to pheromones</td>
</tr>
<tr>
<td>4.9</td>
<td>Correlations between PCNA/Sox2 and percent response to TCA</td>
</tr>
<tr>
<td>4.10</td>
<td>Correlations between calretinin and percent response to TCA</td>
</tr>
</tbody>
</table>
List of Figures (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4.1</td>
<td>Representative confocal images of week 4 immunohistochemistry of olfactory</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>epithelia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4.2</td>
<td>Representative confocal images of wee 16 immunohistochemistry of olfactory</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>epithelia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4.3</td>
<td>Correlations between PCNA/Sox2 and percent response to amino acids</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4.4</td>
<td>Correlations between $G_{a3}$ and percent response to amino acids</td>
<td>155</td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION

Arsenic in the environment

Arsenic (As) is a naturally occurring, ubiquitous metalloid that is found in the air, groundwater, and soil. It is commonly found as inorganic arsenite ($\text{As}^{\text{III}}$) or arsename ($\text{As}^{\text{V}}$), of which arsenite is the more toxic form due to increased cellular uptake and protein interactions (Rehman & Naranmandura, 2012). Organic arsenical species such as arsenobetaine, arsenochochine, and arenosugars are also common, but are typically not as toxic as the inorganic forms. When inorganic arsenite is ingested in mammals, it is absorbed directly through the gastrointestinal tract, then metabolized in the liver by arsenic methyltransferase (As3MT) with the help of glutathione (GSH) and S-adenosylmethionine (SAM) to produce monomethylarsonic acid ($\text{MMA}^\text{V}$), which is further reduced to monomethylarsonous acid ($\text{MMA}^{\text{III}}$) and methylated to produced dimethylarsinic acid ($\text{DMA}^\text{V}$) for removal (Hughes, 2002; Rehman & Naranmandura, 2012; Watanabe & Hirano, 2013).

Arsenic exposure can occur through oral, dermal, and respiratory routes, with oral exposure being the most common. In addition to natural sources, arsenicals can be introduced into the environment through industrial pollution, mining of various ores, and usage of pesticides/herbicides/fertilizers, as well as in poultry and cattle additives (Y.-S. Hong, Song, & Chung, 2014; Mandal & Suzuki, 2002). Although arsenic is no longer used in excess in pesticides and herbicides, it was a very common additive in the 1980s for rice paddies, which remains a contamination issue still today (Meharg & Rahman, 2003; Singh, Singh, Parihar, Singh, & Prasad, 2015). Through both natural and anthropogenic causes, arsenic can contaminate drinking water and food sources, leading to potential long-term exposure for human consumption.
Arsenic is currently listed as the highest ranked toxicant on the ATSDR 2015 substance priority list, and is also classified as a Group 1 human carcinogen (Agency of Toxic Substances and Disease Registry, 2017; Naujokas et al., 2013). The current World Health Organization maximum allowable limit for arsenic in drinking water is 10 ppb, after being reduced from 50 ppb in 2002 (Bloom, Surdu, Neamtiu, & Gurzau, 2014; Naujokas et al., 2013). However, it is estimated that 60-100 million people are exposed to arsenic-contaminated drinking water above these limits daily. Countries such as Bangladesh, India, China, Vietnam, Taiwan, and some regions of the United States have extremely high levels of naturally-occurring arsenic in groundwater, with many areas greatly exceeding regulatory guidelines and reaching as high as 8,000 ppb (Kapaj, Peterson, Liber, & Bhattacharya, 2006; Mandal & Suzuki, 2002; Ng, Wang, & Shream, 2003; Smedley & Kinniburgh, 2002; Smith & Steinmaus, 2009). For example, Bangladesh is commonly referred to as the largest mass poisoning event in history, where almost 50% of the population is exposed to levels well above the World Health Organization limit of 10 ppb (Fendorf, Michael, & Geen, 2010; Kile et al., 2015). Further, arsenic levels over 1,300 ppb have been reported in areas surrounding the Mekong River Delta in Vietnam (Merola, Hien, Quyen, & Vengosh, 2015). Even in areas of the United States such as New England and the southwestern states, arsenic levels as high as 3,000 ppb have been found in private wells (Naujokas et al., 2013). While such high levels of arsenic in drinking water raise major concerns for arsenic-induced toxicities, remediation remains limited due to financial and efficiency restrictions.

In addition to drinking water contamination, dietary arsenic is also a large contributor to long-term exposures. Foods such as rice and rice products, vegetables, fish, and apple juice are typically associated with such exposures due to contaminated irrigation water or previous pesticide use. Rice and rice products produced in areas of high environmental arsenic levels are of
significant concern, especially due to its relevance as a staple food source. The recommended limit of arsenic in food set by the Food and Agriculture Organization (FAO) is 1 mg/kg/day (Singh et al., 2015), and arsenic levels are typically higher in brown rice than in white rice (Meharg, Lombi, et al., 2008). In countries such as Bangladesh, high levels of arsenic in groundwater and irrigation water have produced rice arsenic levels of 160-580 µg/kg, with some reports of levels above 1,800 µg/kg, which produced a daily dietary intake of arsenic of 56.4 µg (M. M. Rahman, Owens, & Naidu, 2009). A supermarket survey in Aberdeen, UK reported that approximately 80% of rice milk samples were over the EU and US standard of 10 ppb, averaging 22 ppb (Meharg, Deacon, et al., 2008). Such high levels of arsenic in a common food staple provides significant sources of exposure, and further support that there are multiple exposure routes of arsenic in daily life.

**Human health effects of arsenic exposure**

Long-term, chronic exposure to arsenic, also described as arsenicosis, is commonly associated with contaminated drinking water and food sources. Such exposures can produce a wide range of health concerns in humans that are both carcinogenic and non-carcinogenic. One of the major indicators of arsenic exposure is keratosis and hyperpigmentation of the skin, which is distinguished by either development and thickening of keratin on the skin or darkening of the skin, respectively. Skin lesions are very common in Bangladesh and West Bengal and typically are one of the first steps for diagnosing chronic arsenic exposure. For example, approximately 22% of the participants in a Bangladesh cohort had melanosis/keratosis skin lesions associated with arsenic-contaminated drinking water (Kapaj et al., 2006). In addition, such skin lesions have been known to develop into basal and squamous cell carcinomas. For example, positive trends were seen in New Hampshire homes between rice intake, urinary arsenic concentrations, and risk
of having squamous cell carcinoma, especially at low levels of arsenic in drinking water (Gossai et al., 2017).

Cardiovascular diseases are also of major concern due to chronic arsenic exposure. Many studies have reported positive associations with arsenic exposure and hypertension, or high blood pressure, in countries such as the United States and Bangladesh (Ratnaike, 2003). One Bangladesh cohort study reported that people exposed to > 148µg/L arsenic were 1.5 times more likely to die from cardiovascular diseases than those exposed to less than 12 µg/L. Also, 28% of heart disease-related deaths were associated with drinking water containing more than 12 µg/L arsenic (Chen et al., 2011). Further, Blackfoot disease is a peripheral vascular disease that is also a distinguishing characteristic of arsenic poisoning, especially in Taiwan (Tseng et al., 2005). The progressive narrowing of blood vessels in the lower extremities eventually leads to tissue necrosis and development of gangrene. In Blackfoot disease endemic regions of Taiwan, strong correlations were reported between peripheral vascular disease and duration of arsenic contaminated well water consumption, cumulative arsenic exposure, and the duration of living the endemic villages (Tseng, Chong, Chen, & Tai, 1996). In addition to peripheral vascular disease, cardiomyopathy and myocardial injury have been associated with chronic arsenic exposure (Y.-S. Hong et al., 2014; Mandal & Suzuki, 2002; Ratnaike, 2003).

Chronic arsenic exposure can also induce a wide variety of effects on the neural system due to the ability of arsenic to successfully cross the blood-brain barrier (Mohammed Abdul, Jayasinghe, Chandana, Jayasumana, & De Silva, 2015). Most commonly associated with chronic exposures is sensory and peripheral neuropathies. In West Bengal, a cohort of 450 patients exposed to > 50µg/L arsenic revealed 37-86% had clinical neuropathies, of which the majority were sensory-related (S. C. Mukherjee et al., 2003). Further, electromyographs reported severe dysfunction of sensory and motor nerves. Many studies have reported that arsenic can directly
cause axonopathy and demyelination of nerves, causing sensorimotor loss (Rodríguez, Jiménez-Capdeville, & Giordano, 2003). In addition, negative associations between arsenic and intelligence, long-term memory, and cognitive function have also been reported in a dose-dependent manner (Y.-S. Hong et al., 2014; Mohammed Abdul et al., 2015). Such intellectual effects are typically common in early life exposures, but chronic exposure exacerbates these effects. For example, full-scale and verbal IQ were negatively associate with urinary arsenic levels in girls exposed both in utero and concurrently (Hamadani et al., 2011). In comparison, West Texas adults exposed to approximately 11µg/L inorganic arsenic in drinking water long-term had poorer global cognition, processing speed, and immediate memory than those without chronic exposure (O’Bryant, Edwards, Menon, Gong, & Barber, 2011). Also, many mechanisms of arsenic toxicity are similar to those seen with neurogenerative diseases and cognitive dysfunction, including induction of oxidative stress and proinflammatory responses, impaired autophagy and protein degradation, and mitochondrial dysfunction (Escudero-Lourdes, 2016).

Inorganic arsenic is also classified as a Group 1 human carcinogen and has been shown to cause skin, liver, lung, bladder, and kidney cancer (Mandal & Suzuki, 2002). Bladder and lung cancer incidences in the United States have been positively associated with 95th percentile arsenic concentrations of 15 µg/L (Mendez et al., 2017). Liver cancer mortality in children in Chile has also been strongly correlated to chronic arsenic exposures (Liaw et al., 2008). Chronic, low-level arsenic exposure has been shown to induce cancer-like phenotypes in normal tissue cells. Human keratinocytes were malignantly transformed and produced cancer stem cell-like characteristics after exposure to 1 µM arsenite (Jiang et al., 2014). Similar results were reported in human peripheral lung epithelial cells, where chronic exposures of 2 µM induced a cancer phenotype (Person et al., 2015). Such mechanistic studies further support arsenic as a potent carcinogen.
**Arsenic as a developmental toxicant**

In addition to the chronic effects of arsenic exposure, the ability of arsenic to negatively impact children during both pre- and postnatal development has recently become a huge focus. Because arsenic can cross the placenta, the fetus can be at an extreme risk for toxic effects during sensitive time points of development. Many epidemiological studies have assessed the effects of arsenic in maternal drinking water and how it impacts proper growth of the fetus before and after birth. What remains unknown is if these adverse effects persist into adulthood once the exposure is removed.

Many studies have reported growth deficits in children due to maternal arsenic exposure. For example, children in Bangladesh that were exposed *in utero* to approximately 100ppb arsenic had birth weights reduced by 168g compared to unexposed children, and had reduced head and chest circumferences (0.05mm and 0.14mm, respectively) per 1ppb AsIII (A. Rahman et al., 2009). Similar results of stunted growth (reductions of 300g) have been seen in girls up to two years of age from postnatal exposures during sensitive times of development (Saha et al., 2012). Further, single pollutant regression models based off of the Flemish human environmental health survey (FLEHS) showed significant correlations between arsenic exposure and lower birth weight (Govarts et al., 2016). Similar effects on growth have also been reported in the United States, West Bengal, China and Taiwan (Bloom et al., 2014; Claus Henn et al., 2016). Significant growth deficits (total body weight) in mice exposed to 10ppb arsenic both *in utero* and postnatally have also been seen (Kozul-Horvath, Zandbergen, Jackson, Enelow, & Hamilton, 2012). Interestingly, cessation of exposure in male mice resolved the growth deficits, whereas resolution did not occur in female mice up to six weeks later.

Based on these epidemiological studies, various laboratory experiments have been initiated to further explore the mechanisms behind such reductions in growth, specifically the impact of
arsenic on early muscle growth and development. Arsenic has been shown to inhibit myogenesis and prevent proper differentiation of muscle progenitor cells into myofibers by blocking the Wnt/β-catenin pathway (G. M. Hong & Bain, 2012). A study by Yen et al. 2010 showed that 0.1-0.5µM arsenic significantly reduced the production of myotubes and myosin heavy chain expression in a dose-dependent manner. They also used mice to investigate arsenic’s effect on muscle regeneration and saw that 0.5 and 5ppm As\textsuperscript{III} in drinking water severely suppressed myogenin expression. If arsenic is reducing the number of myofibers formed by blocking/impairing proper myogenesis, lower birth weight and restricted weight gain may be indicators of this toxicity. Studies in killifish have further supported this reduced weight gain. Fish exposed embryonically to 0-5ppm As\textsuperscript{III} were 8-13% smaller at 16 weeks old compared to controls, and muscle fiber density decreased by 14.3-26.2% compared to controls (D’Amico, Gibson, & Bain, 2014). Further studies showed that embryonic-only exposures shift the muscle fiber size towards smaller fibers (4-9mm diameter) and larger diameter fibers were reduced by 1.6-1.75-fold (Gaworecki, Chapman, Neely, D’Amico, & Bain, 2012). It is clear that arsenic has serious implications during embryonic development and has the ability to disrupt proper muscle growth.

In addition to targeting muscle growth and development, many studies have correlated pre- and postnatal arsenic exposure with neurotoxicity. Arsenic can not only cross the placenta, but also penetrates through the protective blood-brain barrier (BBB) (Mohammed Abdul et al., 2015; Tolins, Ruchirawat, & Landrigan, 2014), which greatly increases risk of neurologic defects during sensitive developmental time points for both the central and peripheral nervous system. \textit{In utero} and early-life exposures to arsenic have been strongly correlated with reduced cognitive functioning, intelligence quotients, working memory, and neurobehavioral disorders. For example, children in Mexico with urinary arsenic (U-As) > 50ppb scored lower on 7 out of 11
cognitive functioning tests compared to children with < 50ppb urinary arsenic (Rosado et al., 2007). Similar correlations between arsenic and reduced cognitive function were reported in Bangladesh (Rodrigues et al., 2016). Developmental arsenic exposure has also shown to reduce both verbal and full-scale IQ in children. *In utero* arsenic exposure that produced 34 ppb and 51 ppb U-As at 1.5 and 5 years of age, respectively, were both significantly associated with both reduced verbal and full-scale IQ tests in girls, and these effects were much more apparent with additional postnatal exposure (Hamadani et al., 2011). Another study also reported reduced intellectual function in Bangladeshi children exposed to > 50ppb arsenic in drinking water compared to children exposed to < 5.5ppb (Wasserman et al., 2004). Further, neurobehavioral changes associated with cognition and relational interactions have been strongly correlated with U-As of 55 ppb in 6-7 year old children in Mexico (Roy et al., 2011).

The exact mechanisms behind arsenic-induced neurotoxicity are still being explored. However, there is very strong evidence that arsenic induces oxidative stress and apoptosis, alters neurotransmitter signaling pathways, and directly impairs development of neuronal cell types (Tolins et al., 2014). Chronic arsenite exposure (25ppm) in the drinking water of rats produced significant decreases in mitochondrial superoxide dismutase activity, an enzyme crucial for the removal of reactive oxygen species (ROS) (Prakash, Soni, & Kumar, 2015). Exposure of mouse neural progenitor cells to 1ppm arsenite for 48 hours significantly increased apoptosis by 51% compared to controls, which was accompanied by increased ROS production (Rocha et al., 2011). Similar results were seen in human neural stem cells, where 1-4µM arsenic produced increased levels of apoptosis, overactivation of JNK and ERK1/2, and decreased PI3K-AKT activity, which all led to reduced neuronal differentiation (Ivanov & Hei, 2013). Arsenic has also been shown to alter levels of dopamine, serotonin, and their metabolites in rat pups exposed through drinking water (Mejía, Díaz-Barriga, Calderón, Ríos, & Jiménez-Capdeville, 1997). Further, spatial
memory and neurobehavioral impairments in rats exposed chronically to 2.72-68ppm arsenite in drinking water were directly associated with 20-29% reductions in N-methyl-D-aspartate receptor (NMDAR) subunit mRNA expression in the hippocampus (Luo et al., 2009). Based on both epidemiological studies and in vivo/in vitro studies, it is clear that arsenic is indeed a neurotoxicant of great concern.

Killifish (*Fundulus heteroclitus*) as a model organism

Killifish (*Fundulus heteroclitus*) are a teleost fish found in brackish waters from northern Maine to northern Florida. They are a common model organism used to study developmental toxicity as they have large, transparent eggs and offspring fully develop in approximately 14-21 days in the laboratory (Burnett et al., 2007). In comparison to rodent models, they can produce a large number of offspring, allowing for a greater statistical power. Further, mouse and rat models require even higher concentrations of arsenic due to differences in arsenic metabolism compared to humans, specifically related to the expression and genetic variation of arsenic methyltransferase (As3MT) (Rehman & Naranmandura, 2012). Killifish have been previously used in our lab to explore the mechanisms behind arsenic-altered myogenesis, and our studies showed that killifish display developmental effects at environmentally-relevant arsenic concentrations that humans are exposed to daily. Further, we have shown that killifish produce similar arsenical metabolites at similar concentrations as humans (in review). Zebrafish (*Danio rerio*) are another frequently used model for toxicity and developmental studies, but they require much higher concentrations of arsenic that are not comparable to human exposures. In some cases, developmental or reproductive effects of embryonic-only arsenic exposures in zebrafish have not been reported below 40ppm (0.5mM AsIII) (D. Li et al., 2009; McCollum et al., 2014).
Because of these reasons, we believe that the killifish is a fitting model organism to examine arsenic-induced alterations in human development.

**Muscle development in mammals**

Arsenic is known to impair muscle development, so it is important to understand how development of skeletal muscle occurs in both mammals and fish. During embryonic development in mammals, skeletal muscles arise from somites produced by paraxial mesoderm early on in the developing embryo (Gilbert, 2013). Skeletal muscle fibers will emerge from the dermamyotome on the most dorsal side of the somite, where it then receives signals from the neural tube and notochord. The major transcription factors involved in myogenesis are myogenic regulatory factors (MRFs), specifically Myf5, MyoD, myogenin, and MRF4 (Florian Bentzinger, Wang, & Rudnicki, 2012). Expression of these transcription factors facilitates the commitment of unspecified tissue to a myogenic lineage. The Wnt signaling pathway plays a crucial role in proper development of the dermamyotome and myotome for myogenesis. Wnt1 is secreted from the dorsal neural tube and its expression leads to increased production of both Pax3 and Myf5 (Florian Bentzinger et al., 2012). Wnt4, Wnt6, Wnt7a are secreted from the ectoderm and work with Pax3 to initiate the production of MyoD. Expression of Pax3 and Pax7 is followed by Myf5 and MyoD, which will eventually lead to the production myogenin and myotubes, followed by mature myofibers (Gilbert, 2013). Sonic hedgehog (Shh) also plays a large role in the progression of myogenesis. It is secreted from the neural tube and notochord and is critical for committed cells expressing Myf5/MyoD that will become mature myotomal cells. In contrast, expression of bone morphogenic proteins (BMPs) leads to inhibition of some myogenic genes (Gilbert, 2013). Spatiotemporal expression of both Wnt and BMPs leads to expansion of the muscle progenitor
cell population followed by commitment to the myogenic lineage. For example, expression of 
*Bmp-4* leads to increased Pax3 expression and decreased Myf5/MyoD expression, which favors 
the undifferentiated state of muscle progenitor cells (Florian Bentzinger et al., 2012). The Notch 
signaling pathway is also highly involved in vertebrate myogenesis, where active signaling 
suppresses MyoD expression and mutations in the Notch ligands Delta1 or RBP-J can produce 
muscle progenitor loss or overactivation of myogenesis (Florian Bentzinger et al., 2012). 
Expression of these MRFs is considered to be terminal in the specification of the myogenic 
lineage of cells.

Following expression of such paracrine transcription factors, fibroblast growth factors (FGFs) 
cause myoblasts to divide until they begin alignment to form myotubes and cell division ceases. 
At this point, expression of integrins, cadherins, and fibronectins begins to aid in the alignment 
process. After alignment of the myoblasts, the fusion process begins with the help of meltrins. 
Myogenin expression follows as an indicator of muscle cell differentiation, and completed fusion 
of myoblasts produces myotubes (Gilbert, 2013). Further growth of the myotubes is supported by 
secretion of interleukin-4 until they become mature muscle fibers.

**Insulin-like growth factor (IGF-1) pathway**

The insulin-like growth factor (IGF) pathway is highly conserved among vertebrates and 
plays a huge role in cell proliferation and growth in the central nervous system, reproductive 
organs, and skeletal muscle development. It consists of multiple IGF ligands (mostly IGF-1 and 
IGF-II), the IGF receptor (IGF-1R, IGF-IIR), and various IGF binding proteins (IGFBP-1 to -6) 
(Sharples et al., 2015). While IGF-1 and IGF-II have similar functions in cellular proliferation 
and growth, their corresponding receptors are functionally and structurally different from each 
other. There are six structurally and biochemically different IGFBPs that all work to regulate the
half-life of IGFs and sequester/transport IGFs when needed (Clemmons, 2016). IGF-1 is produced in the liver in response to various factors, most commonly growth hormone (GH). It is then secreted into the bloodstream, where it is bound to IGFBPs for transport to target tissues such as skeletal muscle. When it arrives at the skeletal muscle fibers, IGF-1 is released from the IGFBP and binds to its receptor, IGF-1R. This initiates phosphorylation of the cytoplasmic subunits and in turn activates signaling cascades for MAPK and PI3K/AKT pathways (Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013; Yu et al., 2015). Activation of such pathways directly leads to cellular proliferation. Local IGF-1 production can also occur in skeletal muscle for autocrine action under periods of stress, injury or reduced IGF-1 secretion (Duan, Ren, & Gao, 2010) to initiate growth pathways.

Many studies have looked at the importance of IGF-1 in relation to growth and development. IGF-1 knockout mice had birth weights of about 40% less than normal mice, and overexpression of IGF-1 has led to increased muscle strength and function (Duan et al., 2010). Negative correlations between plasma IGF-1 levels and arsenic exposure were seen in a cohort study in Bangladesh, and children with lower levels of IGF-1 typically had stunted growth and reduced weight (Ahmed et al., 2013). In addition, IGF-1R knockout mice weighed 55% less than their counterparts and died shortly after birth (Duan et al., 2010).

**Muscle satellite cells**

Muscle satellite cells are stem cells produced from the same central dermamyotome from somites as skeletal muscle. They are undifferentiated stem cells located between the cell membrane and the basal lamina of muscle fibers in a specific stem cell niche, where they remain in a quiescent, inactive cell cycling state (G0 phase) until they are needed for muscle repair/regeneration during adulthood. Satellite cells typically express high levels Pax3 and Pax7,
which inhibits MyoD expression and prevents them from becoming differentiated (Y. X. Wang, Dumont, & Rudnicki, 2014). Lack of Pax7 expression leads to a loss of self-renewal and increased differentiation of satellite cells towards myogenesis (Dick et al., 2015). They can asymmetrically divide to produce identical daughter stem cells for renewal, and also produce committed cells to form new muscle fibers (Gilbert, 2013). Satellite cells play key roles in muscle regeneration in adulthood. Injury or exercise can stimulate these cells to enter the cell cycle, most commonly through expression of growth factors like FGF2 and HGF (Dumont, Bentzinger, Sincennes, & Rudnicki, 2015). Other satellite cell activators include nitric oxide, which is generated immediately following injury that leads to the release of TNF-α and activation of the NF-kB pathway. Activation of satellite cells is also greatly enhanced by high levels of IGF-1 (Bikle et al., 2015). This induces decreased expression of Pax7/Pax3 and increased expression of Myf5 and MyoD to initiate differentiation into myoblasts and myocytes. Further differentiation into myotubes and mature muscle fibers follows with expression of myogenin and myosin heavy chain, respectively.

Proper organization and composition of the stem cell niche is critical for maintenance of satellite cell populations and functions. The stem cell niche provides structural components for satellite cells, such as collagen VI, laminins, fibronectin, glycosaminoglycans, and various proteoglycans, that provide a stable, cross-linked network (Thomas, Engler, & Meyer, 2015). Disorganization of the extracellular matrix can lead to impaired satellite cell function. For example, when naïve human stem cells were transplanted into arsenic-exposed mice muscle tissue, increased fibrosis and disrupted extracellular matrix remodeling was seen (Zhang et al., 2016). Because of this, there was a significant decrease in the myogenicity of muscle satellite cells that directly impaired muscle regeneration. Further, depletion of extracellular matrix proteins (collagen VI) in mice led to reduced stem cell self-renewal and impaired muscle
regeneration in response to skeletal muscle injury (Urciuolo et al., 2013). Disruption of MRF expression in satellite cells can also have severe consequences on muscle regeneration. For example, MyoD/Myf5 double knockout satellite cells lost complete function and were unable to differentiate, thereby impairing muscle regeneration and producing a fibroblast-like phenotype (Yamamoto et al., 2018).

Muscle development in fish

In teleost fish, early myogenesis is very similar to that of mammals. Somites are produced from paraxial mesoderm in a rostral to caudal direction. Muscle progenitor cells, originally expressing Pax7, are similarly committed for myogenic lineage by expression of MyoD and Myf5, then proliferate and differentiate based on expression of MRFs such as myogenin, myogenic regulatory factor 4 (Mrf-4), and myocyte enhancer factor 2 (Mef-2) (Fuentes, Valdés, Molina, & Björnsson, 2013). Expression of myostatin II works against muscle growth to prevent excessive production of muscle fibers (Johnston, 2006). Following the production of myoblasts and fusion into myotubes, the myotubes further fuse and form one of three types of mature muscle fibers: slow (red), intermediate (pink), and fast (white) (Koumans & Akster, 1995). This early embryonic myogenesis produces slow muscle fibers first in a rostral to caudal pattern from somites, which then migrate laterally to lay down a morphogenetic pattern for fast muscle fibers (Johnston, 2006). Further muscle development occurs through stratified hyperplasia, or the addition of slow and fast muscle myotubes in various germinal zones. Hyperplasia occurs during both embryogenesis and continues into the juvenile stage and is the main mechanism of muscle growth after hatching. Mosaic hyperplasia, or the addition of fibers to the surface level of muscle mass, produces many of the fast muscle fibers and is the main mechanism for expanding fast
muscle fiber numbers in juvenile and adult fish. Because of the differences in development of various muscle fiber types, muscle growth in fish is typically a combination of both hypertrophy, a growth of existing fibers, or by hyperplasia, an increase in muscle fiber number (Stickney, Barresi, & Devoto, 2000). Hypertrophy usually occurs in post-embryonic stages after the fish has grown to ~40-50% of its maximum size (Koumans & Akster, 1995). In juvenile and adult fish, most growth consists of hypertrophy, where satellite cells produce new muscle fibers that fuse to existing fibers to promote growth.

**IGF-1 in fish**

Similar to mammals, the IGF signaling pathway is one of the major growth pathways in teleost fishes. However, many teleosts have four IGF ligands (IGF-1, IGF-2A/2B, and IGF-3) and two IGF type 1 receptors, whereas mammals have 2 types of ligands and two receptors (J. Li, Wu, Liu, Wang, & Cheng, 2014). IGF-1 is orthologous to the human IGF-1 and is highly conserved among vertebrates for muscle development and weight gain. IGF-2A/B in teleosts are also orthologous to the human IGF-2, which is a highly expressed fetal growth factor during organogenesis. IGF-3 in fish is unique compared to the other IGF ligands and is mostly known to be expressed in the gonads of adult fish (Fuentes et al., 2013). IGF-1/2 in fish are the most relevant ligands to humans, as they are both essential for growth and are thought to be the main ligands that control and regular muscle fiber mass (Johnston, 2006).

In fish, release of GH from the pituitary gland results in its binding to GH receptors in the liver. This initiates synthesis of IGF-1, which is then released into the blood stream for transport to target tissues while bound to one of three IGFBPs (Fuentes et al., 2013; Johnston, 2006). It is thought that GH is also the main inductor of IGF-1 expression in fish as it is in humans (Moriyama, Ayson, & Kawauchi, 2000). It has been shown that presence of IGFs can increase
protein synthesis and myoblast proliferation in fish species such as trout, seabream, and gulf killifish (Fuentes et al., 2013). And, while studies are much stronger in mammals, both liver-derived and locally-produced IGF seem to be involved in skeletal muscle growth in various fish species. To further support the importance of the IGF pathway for growth in fish, it has been shown that expression of IGFRs is much higher than that of insulin receptors, and similar growth pathways are activated in comparison to mammals (Mommsen, 2001).

**Muscle satellite cells**

Muscle satellite cells serve very similar purposes in teleost fish as they do in humans. They are similarly produced early on in development from the dermamyotome of somites. Developmental studies in zebrafish have shown that the pool of muscle progenitor cells exists under the basal lamina of muscle fibers and remain in a quiescent state while expressing Pax7 and Pax3 until activated (Seger et al., 2011). Expression of MRFs such as MyoD, Myf5, and myogenin are indicative of commitment to the production of myoblasts and eventually myofibers and are typically upregulated in response to injury. While they play key roles during embryonic development, the main function of satellite cells in fish is muscle growth and repair during adulthood. A study comparing muscle generation in zebrafish and sea bream reported that while their mechanisms of normal hyperplasia were different, both species highly relied on the activation and function of satellite cells to repair skeletal muscle tissue (Rowlerson, Radaelli, Mascarello, & Veggetti, 1997). Analysis of Pax7-expressing cells in various fish species have further supported the existence and importance of muscle satellite cells for skeletal muscle repair (Koumans & Akster, 1995; Seger et al., 2011; Zhu et al., 2014).

Because hyperplasia occurs throughout the lifetime of most fish species, satellite cells play important roles in production of new small muscle fibers. Growth of preexisting muscle fibers
also occurs through proliferating muscle progenitor cells, where myoblasts are absorbed into muscle fibers to increase length and diameter (Johnston, 2006). Based on immunohistochemical analyses, new, small muscle fibers express different isotypes of myosin compared to older, larger fibers, but similar isoforms to those of young fibers. This indicates the potential of new muscle fibers being produced from muscle satellite cells (Koumans & Akster, 1995).

**Olfactory development in mammals**

In addition to its effects on muscle growth and development, exposure to arsenic can also lead to severe consequences during neurodevelopment. The embryonic/fetal nervous system is extremely sensitive to toxicants, increasing the probability of malformations and dysfunctions. Early on in development of the nervous system, the ectoderm receives signals from the mesodermal layer (BMP antagonists, Wnt, Fgf) that initiates neural induction to form the neural plate (Schmidt, Strähle, & Scholpp, 2013). Other intrinsic factors, such as Sox2, are crucial for producing and maintaining neural progenitor cells. The neural tube then develops from the neural plate after neurulation occurs, where asymmetrically dividing cells are found in large populations to produce both neurons and more progenitor cells. Transcription factors such as neurogenin1 and Asc1 are found concentrated in proneural cells. Cells with higher concentrations of these factors become further differentiated into neuroblasts (Schmidt et al., 2013), whereas cells with Sox2 or *hairy* expression (i.e. Her4) remain as neural progenitors. Wnt expression typically controls the anterior-posterior patterning of the neural plate and acts as a signal to help dictate positioning of both progenitor and proneural cells. The dorsal-ventral axis is patterned by expression by Shh from the notochord and TGF-β from the dorsal ectoderm (Gilbert, 2010). The neural tube becomes segmented into the telencephalon (containing the olfactory system), diencephalon,
mesencephalon, metencephalon, and myelencephalon. The neuroepithelial cells located in the neural tube give rise to either ependymal cells, neuron precursor cells, or glial precursor cells.

Neurogenesis in the olfactory system occurs both during embryonic development (primary and established neurogenesis) and during adulthood for regeneration of damaged neurons. Embryonic development of the peripheral sensory neurons in the olfactory bulb and epithelium arises from the neural crest and the cranial placodes of the ectoderm. In mice, this typically occurs around E10 in mice. Induction of the olfactory placode regions occurs with expression of *Six3, Pax6,* and *Otx2,* which is quickly followed by the formation of layered structures and neurogenesis (Maier, Saxena, Alsina, Bronner, & Whitfield, 2014). Neurogenic and non-neurogenic fates are then determined. Expression of BMP in maturing olfactory placodal cells leads to an epithelial non-neurogenic fate. *FGF8* is vital for commitment into sensory olfactory epithelial cells, the maintenance of the neurogenic region, and for its role as an antagonist for BMP signaling (Kawauchi, 2005). *Hes1, Sox2,* and *Oct1* also play roles during the production of neurogenic regions in development of the olfactory epithelium and bulb. *Sox2* expression is required to maintain neurogenesis. *Hes* genes have dual roles, working to produce neurogenic regions at early developmental stages but later negatively regulate neurogenesis. *Ascl1* is another gene that marks neural progenitor cells that is required for later differentiation of olfactory sensory neurons (OSNs). 

*Neurog1* and *NeuroD* genes follow *Ascl1* expression for further differentiation into OSNs. Expression of *NeuroD, Runx1,* and *Lhx2* promote expression of NCAM and the production of mature OSNs (Maier et al., 2014).

In mammals, the main structures of the olfactory system are the olfactory epithelium and olfactory bulb. There are two main types of olfactory epithelial cells: basal ciliated and apical microvillus cells. Ciliated cells typically respond to volatile odorants, whereas microvillus cells respond to pheromones and amino acids. Each OSN typically expresses one odorant receptor, and
there are thousands of different odorant genes in humans. Five different transmembrane G-protein coupled receptors (GPCRs) have been distinguished in mammals: odorant receptors (OR), trace amine-associated receptors (TAAR), formyl peptide receptors (FPR), and two vomeronasal receptors (V1R, V2R), with ORs having the largest number of genes (approximately 1,000-1,300) (Kaupp, 2010; Lledo, Gheusi, & Vincent, 2005). Odorant chemicals are initially sensed through the olfactory epithelium, where they activate the corresponding receptors on an OSN. This most commonly initiates the cAMP signaling pathway, but other pathways such as IP3 and cGMP can also be activated (Lledo et al., 2005). This signal is then transferred to the olfactory bulb via sensory neuron projections from the OSNs. These OSN axons innervate one of many glomeruli located in the olfactory bulb. Within the glomeruli, OSN axons form synapses with mitral cells, periglomerular cells, and tufted cells for processing of the olfactory signals. The axons of these cells converge into the olfactory tract, which sends olfactory information to other areas of the brain for processing, such as the frontal lobe and the dorsomedial region of the temporal lobe (Huart, Rombaux, & Hummel, 2013). Due to the constant exposure of the olfactory epithelium to external chemicals and toxins, damage to the OSN population occurs throughout adulthood. Regeneration of epithelial OSNs and supporting cells is therefore necessary throughout adulthood as well. Neurogenesis in the olfactory epithelium is facilitated by progenitor cells located in the basal layers (Brann & Firestein, 2014; Huart et al., 2013). Neurogenesis in adulthood also occurs within the supraventricular zone of the lateral ventricle, which leads to the production of new neuroblasts that can then migrate into the olfactory bulb and differentiate into mature olfactory interneurons (Huart et al., 2013).
Olfactory development in fish

Neurogenesis in teleost fish species is very similar to that of mammals. Zebrafish neurogenesis is used frequently as a model for adult neurogenesis and regeneration. Initially, the mesoderm involutes and contacts the ectoderm during gastrulation. During this process of neural induction, extrinsic factors such as BMP, Wnt, and Fgf and intrinsic factors like those from the SoxB1 family are released for induction or inhibition of neural tissue development around 10 hours post-fertilization (hpf) (Schmidt et al., 2013). Specifically, Noggin and Chordin of the BMP family work to create a permanent neural fate in the dorsal ectoderm, followed by Cerberus and Fgps to fully induce development of the neural plate. Intrinsic SoxB1 members such as Sox 1, 2, 3 and 19 in zebrafish are crucial for neural ectodermal specification. Sox2 in particular is necessary for maintenance of neural progenitor cells both during embryonic development and in adult neural tissue (Schmidt et al., 2013). Following neural plate specification, development of the neural tube occurs through neurulation and asymmetric division of cells. Daughter cells produced apically become neurons, whereas cells produced on the basal side of the neural tube expressing high levels of Notch produce more progenitor cells. Interkinetic nuclear migration follows, where dividing progenitor cells migrate from the apical side to the pial side of the neural epithelium for neurogenesis.

Neurogenesis truly begins with the expression of early proneural genes such as the bHLH neurogenin1 and ascl1 around 20 hpf. At this point, only neural progenitor cells will express SoxB1 family genes. Other progenitor cell pools express hairy genes such as her5 and her11 that block expression of proneural genes and are specific for the development of the midbrain-hindbrain boundary (MHB) (Schmidt et al., 2013). Cells expressing higher levels of proneural genes and lower levels SoxB1 genes become neuroblasts that will further differentiate. Next is the development of the anterior-posterior (AP) axis of the neural plate, which is mostly defined by
gradient expression of Wnt but also with the help of Fgf and Notch proteins. Further, the organization of the mid-diencephalic organizer (MDO) occurs through expression of shh for development of the thalamic complex located in the diencephalon. Wnt expression later becomes restricted to the MDO, MHB, and the dorsal midline. Further neurogenesis in the diencephalon occurs through neurogenetic gradients. Initially, Shh expression from the MDO leads to the induction of more proneural genes like neurog1 (Schmidt et al., 2013). Expression of Her6 then represses neurog1 expression in an anterior-to-posterior fashion, followed by increased expression of delta1 and neurog1 in the neural tube ventricular and subventricular zones. These gradients lead to the production of immature, post-mitotic neurons expressing id2a, lhx2b, hx9, and HuC neuronal markers from 30-42 hpf. Eventually these cells will migrate and develop into mature neurons with specific neurotransmitter properties.

The olfactory epithelium arises from placodes in the developing ectoderm and is highly dependent on the presence of transcription factors such as dlx3b/4b,eya1, and six1b/4b in the pre-placode region. These factors help initiate production of otic and olfactory placode cells (Miyasaka et al., 2013). Fgf signaling in addition to antagonistic BMP and Wnt signaling plays major roles in the specification of the pre-placode region. Placode assembly is highly reliant on expression of the chemokine Cxc12a and its receptor, Cxcr4b. Neural crest cells can then migrate in a posterior to anterior fashion into the placode to join with olfactory placode cells to begin proliferation during early stages of segmental head development. After convergence of placode and neural crest cells, olfactory sensory neurons (OSNs) begin to develop in the olfactory placode. Differentiation into OSNs is strongly dictated by Foxg1, which also controls downstream expression of proneural basic helix-loop-helix (bHLH) genes neurog1 and neuroD4 and is important for the number of differentiated OSNs present (Miyasaka et al., 2013).
Following differentiation into OSNs is the selection for olfactory receptor genes, and in zebrafish it has been proposed to occur in the following steps: selection of an olfactory receptor subfamily cluster, selection of a specific olfactory receptor gene within the cluster, and silencing of other olfactory receptor genes (Miyasaka et al., 2013). OSNs then develop pioneer axons from young neurons that lay the scaffold foundation for navigation to the olfactory bulb. Extensive networking follows for the development of mature axons and their exit from the olfactory placode for olfactory bulb innervation via Cxcl12/Cxcr4, Slit/Robo, and Netrin/DCC signaling.

Neuroblasts also migrate to the olfactory bulb from the subventricular zone, where they then differentiate into GABA inhibitory interneurons, which are important for output processing of the olfactory signals. Once to the glomerulus, OSN axons interact with mitral cell dendrites, which then pass the signal to the diencephalon and telencephalon.

OSNs typically follow a “one neuron-one receptor” method and can express one of three types of G-protein coupled receptors: olfactory receptors (ORs), vomeronasal receptors (V1R, V2R), and trace amine-associated receptors (TAARs). OSNs are typically one of three morphologically different cell types: ciliated (long dendrites), microvillus (microvilli and short dendrites), or crypt cells (pear shaped with microvilli and few cilia) in the olfactory epithelium (Kermen, Franco, Wyatt, & Yaksi, 2013). Crypt cells are unique in fishes in that they are not found in mammalian olfactory epithelia (Bazáes, Olivares, & Schmachtenberg, 2013). These cell types are present in a pseudo-stratified gradient, with ciliated cells being localized in the deep layer of the epithelium, followed by microvillus cells in the intermediate region and crypt cells in the superficial layer. Sensory function of ciliated and microvillus cells usually responds to amino acids, whereas ciliated cells can also sense bile acids and alarm odorants. Crypt cells may be involved in pheromone sensing, although studies have not completely confirmed this (Kermen et al., 2013). Within the olfactory epithelium, a neural stem cell pool exists in the deepest layer for
the constant renewal of ciliated, microvillus, and crypt cells throughout adulthood (Kermen et al., 2013).

The olfactory system is unique in that there is constant neurogenesis throughout adulthood, and because of this it is a great model for adult stem cell renewal and function. There are few studies investigating the impacts of arsenic on the sensory function of the olfactory system. In West Bengal, women exposed to approximately 30 ppb arsenic via drinking water had significantly reduced sense of taste and smell, in addition to tingling and numbness in their extremities (B. Mukherjee, Bindhani, Saha, Sinha, & Ray, 2014). In rodent models, prenatal arsenic exposures produced developmental delays in sensory-motor reflexes in rat pups, indicating impairment of sensory and locomotor development (Gumilar, Lencinas, Bras, Giannuzzi, & Minetti, 2015). While few studies exist that explore the olfactory-mediated behavioral and neurotoxic effects of arsenic in fish, many studies have seen sensory impairments following cadmium, copper, and zinc exposures. For example, 24-hour exposures to cadmium and zinc led to decreased responses to taurocholic acid (TCA) and L-cysteine for both metals (Heffern, Tierney, & Gallagher, 2018). In addition, brief exposures to cadmium produced high levels of cell death within the olfactory placode, which later led to reduced predator avoidance in juvenile fish (Blechinger et al., 2007). Acute copper exposures had similar outcomes, where increased levels of apoptosis and decreased expression of olfactory sensory neurons were seen in salmon, ultimately impairing olfactory signal transduction pathways (L. Wang, Espinoza, & Gallagher, 2013). Because of the known neurotoxic effects of arsenic and the lack of studies investigating its effects on the development and function of the olfactory system in fish, it is important to explore whether embryonic arsenic exposures produce similar effects on the olfactory system as do other metals.
Dissertation Goals and Objectives

The goal of this dissertation is to determine whether low levels of embryonic-only arsenic exposure permanently alters the differentiation and function of stem cells later in life. Specifically, I will be examining two types of adult stem cells - muscle satellite cells and olfactory progenitor cells – and determine whether their alterations will thereby affect skeletal muscle growth and proper functioning of the olfactory epithelium. Previous studies have shown that embryonic-only exposure to 25ppm arsenite significantly altered skeletal muscle fiber organization and reduced muscle fiber size in hatchling killifish (Gaworecki et al., 2012). Further, embryonic-only exposure to 800ppb arsenite significantly reduced growth in killifish up to 16 weeks post-exposure (D’Amico et al., 2014). Therefore, I propose to investigate if stem cells involved in myogenesis and neurogenesis are targeted by arsenic in the killifish model. I hypothesize that embryonic-only exposure to environmentally-relevant levels of arsenic impairs the function stem cells involved in myogenesis and neurogenesis even after the exposure has been removed. My goal will be achieved by three objectives:

Objective 1: Determine whether embryonic arsenic exposure reduces muscle satellite cells in killifish up to one year in age. Effects on growth will be analyzed by using measurements of weight and length at 16, 28, 40, and 52 weeks of age. Condition factors (weight-to-length ratio) will be used to assess the overall growth of the fish. Muscle fiber density is one potential mechanism to correlate to decreased weight gain, which will be quantified using hematoxylin and eosin (H&E). IGF-1 levels have been associated with growth pathways, so quantifying this transcription factor may be connected to changes in growth.
**Objective 2: Determine if arsenic inhibits muscle regeneration after an induced injury.**

Skeletal muscle damage induced by cardiotoxin injections has been well studied in mice to study the regeneration of injured tissue. If arsenic is inhibiting the proper function of muscle satellite cells, I hypothesize that skeletal muscle regeneration after an injury will be reduced in embryonic exposure groups in comparison to the controls. Muscle damage and recovery over time will be assessed using collagen staining for fibrosis formation. In order to determine the differentiated state and function of muscle satellite cells, transcriptional markers such as PCNA (proliferating stem cells) and myogenin (myotubes) will be quantified through IHC. This will help distinguish how many stem cells are present and capable of differentiating.

**Objective 3: To assess the effects of embryonic arsenic exposures on neural stem cells involved in olfactory bulb development.** I hypothesize that embryonic-only arsenic exposures impair neural stem cells during development, producing altered behaviors in killifish due to improper development and differentiation of the olfactory epithelium. Video analysis of killifish swimming behavior after amino acid, bile salt, and pheromone odorant administration will be analyzed by time to start responding, duration of response, and percent response of killifish. These results will then be correlated with growth data. Using IHC, neural stem cell markers such as Sox2 (multipotent stem cells) will be used to quantify the neural progenitor cell populations, in addition to ciliated cells (calretinin) and microvillus cells (Gαi3).
References


CHAPTER TWO

EMBRYONIC-ONLY ARSENIC EXPOSURE IN KILLIFISH (FUNDULUS HETEROCLITUS) REDUCES GROWTH AND ALTERS MUSCLE IGF LEVELS ONE YEAR LATER

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Abstract

Arsenic is a contaminant of drinking water and crops in many parts of the world. Epidemiological studies have shown that arsenic exposure is linked to decreased birth weight, weight gain, and proper skeletal muscle function. The goal of this study was to use killifish (Fundulus heteroclitus) as a model to determine the long-term effects of embryonic-only arsenic exposure on muscle growth and the insulin-like growth factor (IGF) pathway. Killifish embryos were exposed to 0, 50, 200 or 800 ppb As\textsuperscript{III} from fertilization until hatching. Juvenile fish were reared in clean water and muscle samples were collected at 16, 28, 40 and 52 weeks of age. There were significant reductions in condition factors, ranging from 12-17%, in the fish exposed to arsenic at 16, 28 and 40 weeks of age. However, by 52 weeks, no significant changes in condition factors were seen. Alterations in IGF-1R and IGF-1 levels were assessed as a potential mechanism by which growth was reduced. While there were no changes in liver IGF-1 transcripts, skeletal muscle cells can also produce their own IGF-1 and/or alter IGF-1 receptor levels to help enhance growth. After a 200 and 800 ppb embryonic exposure, fish grown in clean water for 16 weeks had IGF-1R transcripts that were 2.8-fold and 2-fold greater, respectively, than unexposed fish. Through 40 weeks of age, IGF1-R remained elevated in the 200ppb and 800ppb embryonic exposure groups by 1.8-3.9-fold, while at 52 weeks of age, IGF-1R levels were still significantly increased in the 800ppb exposure group. Skeletal muscle IGF-1 transcripts were also significantly increased by 1.9-5.1 fold through the 52 weeks of grow-out in clean by water in the 800ppb embryonic exposure group. Based on these results, embryonic arsenic exposure has long-term effects in that it reduces growth and increases both IGF-1 and IGF-1R levels in skeletal muscle even 1 year after the exposure has ended.

Key words:
Arsenic; killifish; embryo; IGF-1; IGF-1R; skeletal muscle
Introduction

Arsenic (As) is a naturally occurring element found throughout the world in the air, water, and soil. It is commonly found as a drinking water and food contaminant, exposing millions of people each year (Mandal and Suzuki, 2002; Ng et al., 2003; Singh et al., 2015). Inorganic arsenic is found in trivalent (III) and pentavalent forms (V), but As\textsuperscript{III} has been found to be the most toxic (Hughes et al., 2011; Lynch et al., 2014; Sharma and Sohn, 2009; Singh et al., 2015). Currently, the WHO standard for arsenic in drinking water is 10ppb, but some countries are still regulated at 50ppb (Ng et al., 2003; Sharma and Sohn, 2009). The main goal of these regulations is to protect people from chronic exposures that cause skin, bladder, and lung cancers in addition to skin, respiratory, and cardiovascular diseases (Hong et al., 2014; Kapaj et al., 2006; Singh et al., 2015). The extent of natural arsenic contamination presents a significant danger of chronic exposure in drinking water in over 70 countries around the world, especially in areas of Southeast Asia (Bloom et al., 2014; Ng et al., 2003; Singh et al., 2015).

While the carcinogenic implications of arsenic are widely studied, there is also a strong correlation between embryonic exposure to arsenic and reductions in growth. Embryonic exposures of arsenic can lead to impaired muscle development and growth (Ambrosio et al., 2014; Yen et al., 2010). For example, children in Bangladesh exposed in utero with 100ppb urinary arsenic had birth weights reduced by 168g compared to unexposed children (Rahman et al., 2009). Two-year-old girls with urinary arsenic levels from 46-96ppb weighed approximately 300g lighter and were 0.7cm shorter (Saha et al., 2012). Mice exposed to 10ppb arsenic both in utero and postnatally weighed 18-23% less than controls at 21 days of age, and these growth deficits continued until 42 days of age, at least in female mice (Kozul-Horvath et al., 2012). Juvenile rainbow trout that consumed *L. variegatus* exposed to 1.5-3.2ppb dietary arsenite weighed 7.4-18.5% less than control fish (Erickson et al., 2010), while exposures of zebrafish
embryos to 2.0mM arsenite produced significant reductions in body weight at 72 and 96 hpf (Li et al., 2009). In locomotor studies, 5ppm arsenite reduced the number of line crossings in zebrafish by 30.5%, and fish exposed to 0.05 and 5ppm spent 23-24% less time in the upper zones of the tank (Baldissarelli et al., 2012).

Killifish (Fundulus heteroclitus) are a useful animal model for developmental toxicity. They have large, transparent eggs, and are fully developed in approximately 14-21 days in the laboratory (Burnett et al., 2007). In comparison to rodent models, they can produce large numbers of offspring, allowing for a greater statistical power. Killifish have been previously exposed to 800ppb either during embryogenesis alone or during embryogenesis along with a 16 week grow period had significant reductions in weight (11-17%), and a 14.3% reduction in muscle fiber density (D’Amico et al., 2014). Exposure of killifish to 25ppm arsenic during embryogenesis revealed significant upregulation of skeletal myosin light and heavy chains expression (2.1- and 1.6-fold, respectively) that was coupled with reduced muscle fiber organization and fewer large muscle fibers (1.6- to 7.5-fold reduction) (Gaworecki et al., 2012).

There are several potential mechanisms by which arsenic is affecting growth and muscle development, including alterations in members of the insulin-like growth factor signaling pathway and impairment of muscle satellite cells. Insulin-like growth factor (IGF-1) is a peptide signal that is produced primarily by the liver in response to increased growth hormone production. IGF-1 is then secreted into and transported by the blood stream, where it binds to IGF-1 receptors on target tissues, such as skeletal muscle, to increase their growth (Bower and Johnston, 2010; Fuentes et al., 2013). IGF-1 knockout mice had birth weights of about 40% less than normal mice, while the overexpression of IGF-1 has led to increased muscle strength and function (Duan et al., 2010). Negative correlations between plasma IGF-1 levels and arsenic exposure were seen in a cohort study in Bangladesh, and children with lower levels of IGF-1
typically had stunted growth and reduced weight (Ahmed et al., 2013). IGF-1 in fish is orthologous to the human IGF-1 and its function is highly conserved among vertebrates for muscle development and weight gain (Duan et al., 2010; Li et al., 2014).

Muscle satellite cells are stem cells produced early in development that remain in skeletal muscle in a quiescent, inactive state. Satellite cells typically express both Pax3 and Pax7, which are transcription factors that prevent satellite cells from becoming differentiated into specific myogenic cells (Swierczek et al., 2015; Wang et al., 2014). Injury can stimulate these satellite cells produce Myf5 and MyoD to initiate differentiation and produce new myofibers (Dumont et al., 2015; Wang et al., 2014). Muscle generation in teleost fish occurs through similar pathways as it does in humans, with skeletal muscle arising from the myotome of somites. In fish, however, the myotubes fuse and form one of three types of mature muscle fibers: slow (red), intermediate (pink), and fast (white) (Koumans and Akster, 1995). Slow muscle fibers typically develop first and eventually lay down a morphogenetic pattern for fast muscle fibers (Johnston, 2006; Johnston et al., 2011). Muscle growth in fish is typically a combination of hypertrophy (the growth of existing fibers) and hyperplasia (the increase in muscle fiber number) (Stickney et al., 2000). Hyperplasia typically occurs during embryogenesis and continues into the juvenile stage to produce both slow and fast fibers. In zebrafish, hypertrophy usually occurs in the post-embryonic stages after the fish has grown to ~40-50% of its maximum size (Koumans and Akster, 1995).

Arsenic has been shown to inhibit myogenesis and prevent proper differentiation of muscle progenitor cells into myotubes by reducing the expression of myogenin and MyoD, transcription factors needed for muscle cell differentiation (Hong and Bain, 2012a). Mice exposed to 0.5 and 5ppm arsenic trioxide in drinking water had reduced myogenin expression and suppressed formation of new muscle fibers after an injury (Yen et al., 2010). If arsenic is reducing the
number of myofibers formed by impairing proper myogenesis, this may manifest itself in reduced
birth weight and weight gain following an embryonic exposure. Indeed, killifish exposed
embryonically to 0-5ppm As\textsuperscript{III} were 8-13\% smaller at 16 weeks old compared to controls, and
their muscle fiber density was decreased by 14-26\% (D’Amico et al., 2014).

The exact mechanisms by which arsenic is reducing growth is still not known, nor is it known
whether the removal of arsenic allows for a recovery. We exposed killifish to arsenic
concentrations of 0, 50, 200 and 800ppb only during embryogenesis, and then after grow-out in
clean water, examined them at 16, 28, 40 and 52 weeks. We found that arsenic significantly
reduced killifish size up to 40 weeks post-exposure and increased skeletal muscle IGF-1 and IGF-1R levels up to 52 weeks post-exposure. These data indicate that embryonic arsenic exposure has
long-term effects on the growth of killifish, which can persist for 1 year after the exposure has
ended.

Methods

Killifish (\textit{Fundulus heteroclitus}) collection and culture.

Adult killifish were collected using minnow traps from the Baruch Institute for Marine and
Coastal Sciences (Georgetown, SC) at the full moon. Killifish were transported to and maintained
in the Aquatic Animal Research Laboratory at Clemson University and kept on a 16:8 light/dark
cycle at 26°C in 18ppt salt water (CoraLife). Killifish were fed TetraMin flake food twice a day
prior to egg collection.
Embryonic exposure of killifish to sodium arsenite.

Milt and eggs from approximately 4 females and 1 male were mixed together to obtain fertilized eggs. The fertilized eggs were then randomly distributed into 28 100mm Petri dishes. This was repeated with 19 more times, with eggs and milt obtained from a total of 80 females and 20 males, until each of the 28 Petri dishes contained approximately 80 eggs. Embryos were exposed to one of four different concentrations of sodium arsenite in 18ppt salt water: 0, 50, 200 or 800ppb (equivalent to 0, 0.67, 2.67, or 10.67μM; n=7 Petri dishes per exposure group). Water changes (25mL) were done every other day, and embryos were monitored daily for viability and hatching. Juvenile fish were removed from Petri dishes within 24 hours of hatching and placed in 10 gallon tanks containing arsenite-free 18ppt salt water. All fish from one Petri dish were transferred to the same tank for grow out. Juveniles were fed brine shrimp for about 4 weeks then gradually switched to TetraMin flake food. Killifish remained in clean water until they were sampled at 16, 28, 40 and 52 weeks of age.

Feeding behavior study

One week before each sampling time point, feeding behavior tests were performed to assess changes in fish position in each tank in response to a food stimulus. The study was conducted in the home tanks, and fish were not fed for 15 hours prior to starting the test. One week prior to starting the behavioral test, a 10’x10’ gridded tile was inserted into each home tank. The grids on the tile divided each tank into five rows, with row 1 being the top of the tank and row 5 as the bottom of the tank. A video camera mounted to a tripod was used to record the tests. The camera was positioned in a slit behind a large cardboard box to shield the observer from the fish. Similarly, the person adding the feed was positioned behind the tanks and behind shielding. Prior
to the start of the test, fish behavior and movement were video recorded for 30 seconds to
document normal behavior and location within the tank. To start the test, Tetramin flake food
was then placed on the top of the water column, and tank activity was recoded for an additional
30 seconds. The total number of fish in each row were counted and used to determine percent
occupancy in each row at 0, 5, 15 and 25 seconds after feeding. Feeding behavior tests were
performed on three separate days, and the results were averaged per tank.

*Growth measurements of killifish*

At each of the four time points, 4-8 fish per tank were euthanized (1g/L buffered MS-222),
depending on the population of each tank. Starting at 28 weeks, equal numbers of males and
females (≥2 per sex per tank) were sampled when possible. Fish were dried to remove excess
water, and weight and length were measured. Condition factors (weight/length³) were calculated
for each fish.

*Muscle fiber density*

Sections of trunk muscle directly posterior to the dorsal fin were collected (4-8 fish per tank;
n=7 tanks per exposure group) and fixed in 10% neutral-buffered formalin for 18 hours,
progressively dehydrated in ethanol, and paraffin-embedded. Blocks were cut into 7µm sections
and one muscle trunk sample per tank at 16 weeks was used for hematoxylin and eosin (H&E)
staining (n=6 fish per exposure group). Once the fish could be sexed, approximately 3 males and
3 females per tank were taken for muscle fiber density quantification at 28, 40 and 52 weeks.
Slides were imaged using a Leica ICC50 HD light microscope and Leica EZ suite software.
ImageJ software (National Institutes of Health) was used to count muscle fibers. Upper right quadrants of each tissue section were selected for quantification and areas were determined (mm$^2$) to calculate density (fibers/mm$^2$). Average densities for all fish and males/females were determined for each tank.

$qPCR$

At each sampling time point, sections of killifish truck muscle directly anterior to the caudal fin were collected from 4-8 fish in each tank. Muscle samples were stored in RNAlater at -80°C. Extraction of RNA was performed using TRIZol (Sigma-Aldrich, St. Louis, MO). Purity and concentration of RNA was determined using a NanoDrop Lite, and the RNA (2µg) was reverse-transcribed to cDNA using MMLV-RT.

Gene specific primers for killifish insulin-like growth factor-1 (IGF-1), insulin-like growth factor-1 receptor (IGF-1R), and 18S rRNA were used to develop purified standards (IGF-1 Forward: AAACAGATAAAACCAACAGCTATG; IGF-1 Reverse: GCAGCTCACAACTCTGGAA; IGF-1R Forward: CGCAGAAATGTACGTACCAGA; IGF-1R Reverse: CGTCTTTGACCACACCCTT; 18S Forward: TTTCTCGATTCTGTGGGTGGTGGT; 18S Reverse: TAGTTAGCATGCGGAGTCTCGTT). To conduct qPCR, 40ng cDNA was incubated with SYBR green (Qiagen, Alameda, CA) on an iQ5 thermocycler (Bio-Rad). For each time point, five males and five females from different tanks of each exposure group were randomly selected for analysis. A 5-point curve (10$^{-3}$ to 10$^{-7}$ng) of purified standard for IGF-1, IGF-1R and 18S rRNA to determine the efficiency and number of molecules in each tissue sample. Amplification of each specific transcript was confirmed by melt curves, and all samples, standards, and no template negative controls was run in triplicate. All standard curves yielded
reactions that were > 90% efficient with $r^2 > 0.98$. The total number of IGF-1 and IGF-1R molecules in each sample were normalized using 18S (housekeeping gene), and then divided by the fish’s wet weight (g) to account for the size of the fish. IGF-1 molecules/g and IGF-1R molecules/g were then averaged by exposure group for all fish, and then for each gender.

**Statistical analysis**

Statistical models for feeding behaviors and percent occupancy were developed in JMP that included terms for the factors exposure level, random tank within exposure level, time, and time by exposure. A two-way ANOVA was used to test the factors, followed by Tukey’s ($p<0.05$). Differences in condition factors, muscle fiber densities, IGF-1R, and IGF-1 levels were assessed by two-way ANOV, followed by Tukey’s ($p<0.05$), using GraphPad Prism 4.

**Results**

*Arsenic exposure during embryogenesis reduces long-term growth of killifish*

Appropriate concentrations of arsenic for an embryonic-only exposure were determined previously (D’Amico et al., 2014). The fertilization success (defined as viable embryos 3 days post-fertilization) ranged from 93-97% and was not significantly different between groups. Exposures up to 800ppb did not affect hatching success as there were no significant differences compared to the control (Fig. 1A). Fish survival through the 52 week study was also not significantly affected by any of the arsenic exposures (Fig. 1B). Condition factors (CF; weight/length$^3$) were used as an index of growth. At 16 weeks, there were significant decreases in condition factors at 200 and 800ppb, with reductions of 12-18% compared to the control fish.
(Fig. 2A). No differences were seen at 50ppb initially. However, by 28 weeks, male fish CFs were significantly reduced by 15-20% at 50 and 200ppb, respectively (Fig. 2B), while the female CFs were reduced by 15-18% at 200 and 800ppb (Fig. 2C). When the genders were combined, there were significant reductions in all three exposure groups compared to the control fish. By 40 weeks of age, however, the statistically significant differences in CF have diminished except that the 800ppb males had 15% reductions in CF. All significant reductions in CF are no longer present by 52 weeks of age (Fig. 2). These data suggest that by 52 weeks of age, killifish embryonically exposed to arsenic are able to compensate for the growth deficits seen earlier on in life.

*Embryonic-only arsenic exposures alter killifish behavior.*

Within the first 3 months of grow out, differences in killifish behavior across exposure groups were observed. Control fish swam independently throughout the tanks and surfaced for food, while 200 and 800ppb fish remained clustered in the bottoms of the tanks and waited for food to sink down to them. Preliminary behavior studies revealed that at 16 weeks of age, exposed fish took up to 13.6-fold longer to reach the surface of the water column in response to food (data not shown). So, we more systematically examined changes in behavior by assessing positioning in the tank during feeding using a 5 row gridded tile and recording the location of each fish over time. At 28 weeks, the percent of 200ppb fish remaining in the bottom of the tank (row 5) after food was administered was approximately 2-fold greater than control fish (Fig. 3A) at all four time points, while the number of fish from the 800ppb group remaining in the bottom of the tank was approximately 1.5-fold greater than controls. At 40 weeks, percent occupancy of the bottom of the tank by the 800ppb fish remained higher during feeding (Fig. 3B). By 52 weeks of age, no significant differences were seen (Fig. 3C).
**Embryonic-only exposure to arsenic does not alter muscle fiber density.**

Muscle fiber density was quantified using cross-sections of killifish trunk muscle to determine if this was a potential cause of the growth reductions. Sections of trunk muscle were stained with hematoxylin and eosin for fiber quantification. There were no overt differences seen in shape or structure of the muscles (Fig. 4). At 16 weeks, the 200 and 800ppb groups had approximately 170% more fibers than the controls (Fig. 5). However, these differences in muscle fiber density did not persist beyond 16 weeks in any exposure group. At 16 weeks, there appeared to be smaller fiber diameters in the 200 and 800ppb groups (data not shown).

**IGF-1 and IGF-1R transcripts are increased after embryonic-only arsenic exposure.**

IGF-1 and its receptor, IGF-1R, are key components in the growth pathways involved in skeletal muscle differentiation and proliferation. We hypothesized that altered levels of these proteins due to embryonic arsenic exposure may play a role in reducing killifish growth. Under normal conditions, IGF-1 is produced and secreted by the liver. However, if an organism is under stress, IGF-1 can also be produced and secreted from skeletal muscle (Bikle et al., 2015; Moriyama et al., 2000). IGF-1R is found in the plasma membrane of skeletal muscle cells, and expression is extremely important for activation of the IGF-1 pathway. At 16 weeks of age, IGF-1R transcript levels are 16- to 29-fold greater than at 28 weeks, likely due to a higher growth rate of the fish (Fig. 6). IGF-1R transcript levels continue to decrease as the fish age. For example, there are 500 IGF-1R mRNA molecules per gram weight of fish at 16 weeks, 27 IGF-1R mRNA molecules at 28 weeks, and 7 IGF-1R mRNA molecules at 40 weeks of age.
Following the embryonic arsenic exposure, when the fish are 16 weeks, IGF-1R levels are significantly higher (~2-fold) in 200 and 800ppb fish (Fig. 6A). At 28 weeks, IGF-1R transcripts are still elevated by 1.8- to 2-fold in the 200ppb fish (Fig. 6A and B), and while the levels are increased in 800ppb fish, this is not statistically significant. At 40 weeks, IGF-1R transcripts are still increased by 2.2- and 3.9-fold in the 200 and 800ppb exposure groups, respectively (Fig. 6). These increases in expression are mostly driven by the male IGF-1R levels, which were increased by 3.8-fold in the 200ppb group, and by 6.1-fold in the 800ppb group (Fig. 6A). Significant increases in females were only seen at 800ppb (2.7-fold increase). At 52 weeks, similar trends were seen, with IGF-1R increased by 1.3-fold in the combined fish at 200 and 800ppb (Fig. 6A) and 1.5-fold in 800ppb females (Fig. 6C).

Next, skeletal muscle IGF-1 transcripts were examined. In control fish, IGF-1 levels were also elevated early on, but decrease as the fish age (Fig. 7A). For example, there are 500 IGF1 mRNA molecules per gram weight of fish at 16 weeks, 42 IGF1 mRNA molecules at 28 weeks, and ~15 IGF1 mRNA molecules at 40 and 52 weeks of age. At 16 weeks, fish exposed to 800ppb arsenic as embryos had 2.9-fold greater expression of IGF-1 (Fig. 7A). By 28 weeks, IGF-1 was 2-3-fold greater in males, females, and both combined (Fig. 7A-C). IGF-1 levels in 800ppb fish was also increased (1.6-fold greater) but was not statistically significant. At 40 weeks, IGF-1 in 800ppb fish (males, females, combined) is still 2.6-5.1-fold greater than controls (Fig. 7A-C). IGF-1 levels in 200ppb fish are not significant but are 2-fold greater than controls in all three group. By 52 weeks, IGF-1 in 800ppb fish are still significantly increased in female fish by 1.9-fold. These results indicate that upregulation of skeletal muscle IGF-1R and IGF-1 skeletal may be a way to compensate and overcome growth deficits seen fish embryonically exposed to arsenic.
Discussion

The results of this study indicate that embryonic-only exposures to environmentally-relevant levels of arsenic (0-800ppb) can reduce the growth of killifish. In an apparent attempt to compensate and gain mass, the embryonically-exposed fish increase their skeletal muscle levels of both IGF-1 and IGF-1 receptor. These effects persist into adulthood and are still seen at 40-52 weeks of age, long after the arsenic exposure has ended. This study indicates that embryonic arsenic exposures can have long term consequences in adults.

*Embryonic-only arsenic exposure reduces growth into adulthood*

A previous study had examined growth in killifish after an embryonic arsenic exposure, by assessing weight in the fish at 2, 4, 8, and 16 weeks after hatching. That study indicated that arsenic reduced weight in the 8 and 16 week old juvenile fish (D’Amico et al., 2014). The current study was designed to examine the persistence of the weight gain reductions by examining the fish up to 1 year of age, at reproductive maturity. When examining growth starting at 16 weeks of age, there were significant decreases of 12-18% in condition factor (growth index) in both 200 and 800ppb embryonically-exposed fish. These results are similar to those seen previously, where embryonic exposures of 800ppb led to an 11% reduction in weight at 16 weeks (D’Amico et al., 2014). These reductions in size continue through 28 weeks of age, are seen in both sexes, and reduction in growth occurs at even lower arsenic concentrations, down to 50ppb arsenic. Growth reductions continued until 40 weeks of age, but only in male fish. Differences in growth between male and female fish may be due to increased weight from presence of eggs in sexually-mature females, which was not accounted for. By 52 weeks, exposed fish are no longer significantly smaller than controls. However, the control fish condition factors were actually
reduced at 52 weeks, while the arsenic exposed fish continued to grow. Why this occurred is unknown, but is particularly notable in the males. At this point in time, spawning season has just passed. It is possible that control fish were expending more energy towards reproduction rather than growth, as the lengths of the fish were similar. Or, it simply may be that exposed fish have caught-up to their control counterparts.

Similar to our findings, several epidemiological studies have noted that embryonic arsenic exposures from maternal drinking water reduces birth weight and weight gain in children (Bloom et al., 2014; Davis et al., 2015; Hopenhayn et al., 2003; Kile et al., 2016). For example, children born to mothers in arsenic-exposed villages in Taiwan were 29g lighter than those from non-exposed villages (Yang et al., 2003). In a study near a Superfund mining site in Oklahoma, maternal blood arsenic was directly associated with significantly lower birth weight, shorter gestational age, and smaller head circumference (Claus Henn et al., 2016), while in a Bangladesh cohort, birth weight was reduced by 1.7g for each 1μg/L increase in maternal urinary arsenic (Rahman et al., 2009). In children who were prenatally exposed to arsenic, weight gain is also reduced. For example, children whose mothers had urinary arsenic levels between 63-120ppb at 30 weeks of gestation had children who weighed 40g less at 6 months, 100g less at 1 year of age, and 120g less at 2 years of age compared to mothers with less than 35ppb urinary arsenic (Saha et al., 2012).

There are also some studies that noticed gender differences with arsenic’s effects on growth. One study found that after prenatal exposures, decreased weight gain was significantly correlated with increased maternal urinary arsenic, and that this trend was more robust in girls (Rahman et al., 2009). However, another study found that only male newborns had strong inverse correlations between birth weight and maternal blood arsenic (Xu et al., 2011), and in rural Bangladesh maternal urinary arsenic was weakly correlated with reduced fetal size in boys.
(Kippler et al., 2012). In our study, most reductions in size were seen in male fish and not female fish after 28 weeks. However, the mass attributed to developing oocytes and eggs in the females at 40 and 52 weeks was not accounted for, this likely added additional weight to the females and also contributed to the increased variability at these ages. Whether there are true gender differences in weight gain after embryonic arsenic exposure remains to be seen.

One potential mechanism by which arsenic may reduce weight gain is by altering skeletal muscle fiber size or density. Although our study did not reveal reductions in fiber density in exposed fish compared to controls, we did observe a slight shift in muscle fiber size to smaller diameter fibers at 16 weeks (data not shown) that did not persist in later time points. Previous studies in our lab also reported embryonic-only exposures of arsenic (800-5000ppb) altered the muscle fiber size distribution, with the 800ppb group showing a slight, but significant reduction in the largest diameter fiber (>10µm) numbers (D’Amico et al., 2014). While the previous study saw reductions in fiber density in exposed fish at 16 weeks, the current study did not see similar results. Both studies saw changes in weight, with the reductions at 800ppb in the current study are slightly greater. The previous exposure also resulted in significant reductions in length (D’Amico et al., 2014). The differences in length might explain why similar changes in muscle fiber density were not seen between studies. Arsenic is also known to impair myogenesis and prevent the proper myotube formation (Hong and Bain, 2012a; Steffens et al., 2011; Yen et al., 2010). However, it is still unclear if reduced skeletal muscle density is a mechanism by which arsenic reduces growth.

*Embryonic-only arsenic exposures alter the IGF-1 signaling pathway*

Insulin-like growth factor-1 (IGF-1) and its receptor, IGF-1R, are key components in the IGF-1 growth pathway that stimulates myoblast proliferation for proper skeletal muscle growth
and development, especially in fish (Johnston, 2006; Johnston et al., 2011). Binding of IGF-1 to IGF-1R leads to the phosphorylation of AKT and mTOR, increasing the expression of muscle-specific transcription factors, such as myogenin, and therefore myoblast differentiation (Fuentes et al., 2013). IGF-1 can also activate p38 MAPK and increase muscle cell proliferation. IGF-1 and IGF-1R isoforms have been shown to be similar across vertebrate species, including humans and fish (Fuentes et al., 2013; Reinecke et al., 2005).

IGF-1 is produced and secreted from the liver, after which it binds to IGF binding proteins (IGFBP) for transportation via plasma to target tissues and to regulate IGF-1 half-life (Duan et al., 2010). The IGF receptor (IGF-1R) is found on target tissues such as skeletal muscle for activation of growth and differentiation pathways and is highly conserved, both structurally and functionally, between fish and mammals (Duan, 1998). Two forms of the IGF receptor, IGF-1Ra and IGF-1Rb, have been identified in zebrafish and salmonids (Reinecke et al., 2005). Although there seems to be differential expression of these receptors during fasting and refeeding, it is not known if they have distinct functions. The main organ for arsenic accumulation is the liver, which increases the likelihood for interaction with the IGF pathway. Because plasma IGF-1 originates from the liver, plasma levels should be indicative of liver function for the IGF pathway. However, under times of stress or impaired liver function, IGF-1 can be produced and upregulated in a paracrine manner from skeletal muscle (Picha et al., 2008). For example, one study saw 40% reductions in IGF-1R mRNA during four days of refeeding (Chauvigne et al., 2003).

Killifish embryonically exposed to 200 and 800ppb arsenic had skeletal muscle IGF-1R and IGF-1 transcripts that were increased through 52 weeks of age. Although the absolute levels of transcripts are slowly reduced as the fish age, at all time points examined, embryonic arsenic exposure increased IGF-1 and IGF-1R transcripts in the skeletal muscle of these fish by approximately two-fold. However, hepatic IGF-1 mRNA levels at 52 weeks were not changed by
embryonic-only arsenic exposure (data not shown). Human epidemiological studies have found a correlation between reduced plasma IGF-1 levels and in utero arsenic exposure in when measured at 4.5 years of age (Ahmed et al., 2013). We did not measure plasma levels of IGF-1 but hypothesize that the upregulation of skeletal muscle IGF-1R was at attempt to increase growth in response to changes in serum IGF-1 levels. Other studies have also seen alterations in IGF-1 protein and transcript levels following arsenic exposure. For example, arsenic exposure to mouse myocytes undergoing differentiation reduced IGF-1 mRNA levels by 2-fold (Hong and Bain, 2012b), while arsenic suppressed plasma IGF-1 levels in prepubertal mice (Reilly et al., 2014).

Upregulation of IGF-1R and IGF-1 in skeletal muscle may be a compensatory mechanism that the fish undergo to overcome deficits in growth. In other organisms undergoing growth restriction due to fasting, skeletal muscle IGF-1 mRNA levels are increased (Hornick et al., 2000). For example, muscle IGF-1 transcripts were increased by 8- to 14-fold during re-feeding of rainbow trout after a 10-week fast, but no differences were seen in IGF-1R mRNA levels (Chauvigne et al., 2003). Similarly, after a 3-week fast in hybrid striped bass, IGF-1 mRNA in skeletal muscle increased approximately 10-fold during refeeding (Picha et al., 2008).

**Conclusions**

In summary, embryonic-only arsenic exposures produce long-lasting reductions in growth that are present up to 1 year of age. Along with changes in growth, skeletal muscle IGF-1 and IGF-1R were significantly and persistently increased by these exposures, potentially as a compensator mechanism. Overall, these results indicate that embryonic arsenic exposure results in growth deficits long after the exposure had ended.
**Conflicts of interest**

The authors declare that no conflicts of interest exist.

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References


Figure 2.1. Hatching success and fish survival of killifish is not altered after embryonic-only arsenic exposure. Hatching success was compared to the initial number of viable embryos (A). Fish survival was determined based on the average number of fish per tank and compared to the average number of fish surviving in the control group (B). Data is presented as mean ± SD with n = 7 tanks for each exposure group. Statistical significance was determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 2.2. Embryonic-only arsenic reduces condition factors of killifish until 40 weeks of age. Weight and length measurements were collected for 4-8 fish per tank for each exposure, and condition factors calculated (weight/length$^3$). Condition factors for each tank were then averaged by tank (n=7) and exposure group. Condition factors are presented for all fish (A), male fish only (B), and female fish only (C). Statistical differences (a = 50ppb, b = 200ppb, c = 800ppb) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 2.3. Embryonic-only arsenic causes an increase in percent occupancy in the bottom of the tank. Fish behavior was recorded for 30 seconds after the addition of food to the top of the water column. Percent occupancy was determined by counting the number of fish in row 5 in relation to the total number of fish in each tank (n=7) and averaged across exposures. Feeding behavior tests were performed three times in one week. Data is presented as percent occupancy over time for 28 weeks (A), 40 weeks (B), and 52 weeks (C). Statistical differences were determined using ANOVA and Tukey’s post-hoc test through a JMP statistical model (p<0.05).
Figure 2.4. Representative images of killifish trunk muscle cross sections. Tissues were formalin fixed, paraffin embedded and stained with hematoxylin and eosin.
Figure 2.5. Arsenic increases muscle fiber density at 16 weeks of age. Cross-sections of killifish trunk muscle were stained with hematoxylin and eosin. ImageJ was used to quantify the upper right quadrant of the cross-section. The total number of fibers in each section was divided by the quantified area to obtain muscle fiber density (fibers/mm²) (16 weeks: n=6 per exposure;
28, 40, and 52 weeks: n=3 males and 3 females per exposure). Average muscle fiber densities are presented as all fish (A), male fish only (B), and female fish only (C). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 2.6. Embryonic arsenic exposure increases skeletal muscle IGF-1R mRNA levels.

Samples of trunk muscle were collected at 16, 28, 40 and 52 weeks of age and IGF-1R transcripts were quantified by qPCR. Five samples at 16 weeks were randomly selected from various tanks of each exposure group to be analyzed. At 28, 40 and 52 weeks, five males and five females from
different tanks of each exposure group were randomly selected for analysis. Each sample was run in triplicate and normalized to 18S. Data is expressed as the average number of IGF-1R molecules per gram of fish weight to account for the size of the fish. Data presented for all fish (A), male fish only (B), and female fish only (C). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 2.7. Embryonic arsenic exposure increases skeletal muscle IGF-1 mRNA levels.

Samples of trunk muscle were collected at 16, 28, 40 and 52 weeks of age and IGF-1 transcripts were quantified by qPCR. Five samples at 16 weeks were randomly selected from various tanks.
of each exposure group to be analyzed. At 28, 40 and 52 weeks, five males and five females from
different tanks of each exposure group were randomly selected for analysis. Each sample was run
in triplicate and normalized to 18S. Data is expressed as the average number of IGF molecules
per gram of fish weight to account for the size of the fish. Data presented for all fish (A), male
fish only (B), and female fish only (C). Statistical differences (*) were determined using
ANOVA and Tukey’s post-hoc test (p<0.05).
CHAPTER THREE

EMBRYONIC-ONLY ARSENIC EXPOSURE ALTERS SKELETAL MUSCLE SATELLITE CELL FUNCTION IN KILLIFISH (FUNDULUS HETEROCLITUS)

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Abstract

Arsenic is a contaminant found worldwide in drinking water and food. Epidemiological studies have correlated arsenic exposure with reduced weight gain and improper muscular development, while in vitro studies show that arsenic exposure impairs myogenic differentiation. The purpose of this study was to use Fundulus heteroclitus or killifish as a model organism to determine if embryonic-only arsenic exposure permanently reduces the number or function of muscle satellite cells. Killifish embryos were exposed to 0, 50, 200, or 800 ppb arsenite (As\textsuperscript{III}) until hatching, and then juvenile fish were raised in clean water. At 28, 40, and 52 weeks after hatching, skeletal muscle injuries were induced by injecting cardiotoxin into the trunk of the fish just posterior to the dorsal fin. Muscle sections were collected at 0, 3 and 10 days post-injury. Collagen levels were used to assess muscle tissue damage and recovery, while levels of proliferating cell nuclear antigen (PCNA) and myogenin were quantified to compare proliferating cells and newly formed myoblasts. At 28 weeks of age, baseline collagen levels were 105% and 112% greater in 200 and 800 ppb groups, respectively, and at 52 weeks of age, were 58% higher than controls in the 200 ppb fish. After cardiotoxin injury, collagen levels tend to increase to a greater extent and take longer to resolve in the arsenic exposed fish. The number of baseline PCNA(+) cells were 48-216% greater in 800 ppb exposed fish compared to controls, depending on the week examined. However, following cardiotoxin injury, PCNA is reduced at 28 weeks in 200 and 800 ppb fish at day 3 during the recovery period. By 52 weeks, there are significant reductions in PCNA in all exposure groups at day 3 of the recovery period. Based on these results, embryonic arsenic exposure increases baseline collagen levels and PCNA(+) cells in skeletal muscle. However, when these fish are challenged with a muscle injury, the proliferation and differentiation of satellite cells into myogenic precursors is impaired and instead, the fish appear to be favoring a fibrotic resolution to the injury.
Key words:
Arsenic; fish; embryo; satellite cell; myogenin; collagen

Introduction

Arsenic (As) is a well-known environmental contaminant that millions of people in over 70 counties are exposed to each day through food and drinking water sources (Ratnaike, 2003; Ng et al., 2003; Singh et al., 2015). Arsenic exists in both trivalent (As\textsuperscript{III}) and pentavalent forms (As\textsuperscript{V}), but As\textsuperscript{III} has been shown to be much more toxic (Hughes, 2002; Sharma and Sohn, 2009). The World Health Organization and Environmental Protection Agency have classified arsenic as a Group 1 human carcinogen and have set recommended levels of arsenic in drinking water at 10 parts per billion (ppb), although a number of countries have limits of 50 ppb. These drinking water standards are put in place mainly for protection from chronic carcinogenic effects as well as skin diseases (Hong et al., 2014).

While these arsenic standards appear to be protective against cancer, it is not well known if these standards also provide protection against developmental toxicities. Arsenic can cross the placental barrier (Punshon et al., 2015), which causes great concern for proper embryonic and fetal development. Many epidemiological studies have correlated arsenic-contaminated drinking water with reduced birth weight and weight gain in children who were exposed prenatally through contaminated maternal drinking water (Mandal and Suzuki, 2002; Kapaj et al., 2006; Almberg et al., 2017). For example, both maternal and cord blood arsenic concentrations were negatively associated with birth weight, gestational length, and head circumference in patients near a mining-related Superfund site in Oklahoma (Claus Henn et al., 2016). Another study reported a 1.68 g decrease in body weight per 1µg/L increase of urinary arsenic after prenatal exposures less than 100 µg/L (Rahman et al., 2009). Animal models show similar effects in that 28 week old
killifish who were exposed to arsenic as embryos were reduced in size by 15% (Szymkowicz et al. 2017), and mice exposed to 10 ppb \textit{in utero} had significantly lower total body weight than controls (18-20% reductions) at 21 days after birth (Kozul-Horvath et al., 2012).

One of the potential mechanisms by which arsenic may be altering muscle development is through changes in the number and/or function of muscle satellite cells. During embryonic development, signals are sent from the neural tube (Wnt1/neurotrophin-3) and the notochord (sonic hedgehog) to the myotome (Florian Bentzinger et al., 2012). These signals initiate the production of Pax3, followed by Myf5 and MyoD, which direct the tissue towards a myogenic lineage (Johnston et al., 2011). Muscle satellite cells are produced from the dermamyotome and remain in a quiescent, inactive state on skeletal muscle fibers until they are needed for muscle repair/regeneration (Florian Bentzinger et al., 2012; Dumont et al., 2015). They are capable of producing both identical daughter cells for renewal and differentiated cells to produce new muscle fibers. Satellite cells express transcription factors such as Pax3 and Pax7, which prevents them from becoming differentiated into myofibers (Fuentes et al., 2013; Dick et al., 2015). However, injury or exercise can stimulate satellite cells to enter the cell cycle to express Myf5 and MyoD to start differentiation (Dumont et al., 2015; Tierney and Sacco, 2016). This leads to the production of transcription factors such as myogenin and myosin heavy chain that will eventually produce mature muscle fibers.

Arsenic is known to inhibit myogenesis. In P19 embryonic stem cells, arsenic concentrations as low as 7.5 µg/L significantly reduced expression of Myf5, MyoD, and myogenin and reduced their differentiation into myotubes (Hong and Bain, 2012). Exposures of 0.1-0.5 µM As$_2$O$_3$ also inhibited myotube formation in mouse C2C12 myoblasts and reduced myosin heavy chain and myogenin expression (Yen et al., 2010). This same study also reported reduced myogenin expression and increase collagen deposits after skeletal muscle injury in mouse soleus muscle
after 8 weeks of exposure through drinking water (0.5 and 5 ppm \( \text{As}_2\text{O}_3 \)). Arsenic has shown to alter mitochondrial morphology and oxygen consumption of muscle progenitor cells after 2-5 weeks of 100 \( \mu\text{g/L} \) drinking water exposures (Ambrosio et al., 2014). Mice exposed to 100 \( \mu\text{g/L} \) in drinking water for five weeks impaired muscle regeneration after injury and produced pathogenic extracellular matrix remodeling, (Zhang et al., 2016). Based on these studies, it is clear that progenitor cells, particularly muscle satellite cells, are affected by arsenic.

Killifish (\textit{Fundulus heteroclitus}) is a commonly used animal model for developmental toxicity studies. Killifish are teleost fish that produce a large number of transparent eggs such that they are fully developed in 14-21 days in a laboratory setting. Previous studies showed that killifish exposed to 800 ppb \( \text{As}^{\text{III}} \) during embryogenesis had significant body weight reductions (11-17%) and reduced muscle fiber densities (14.3%) (D’Amico et al., 2014). Other studies showed that 25ppm arsenite exposures during embryogenesis led to upregulated skeletal myosin light and heavy chain transcripts (2.1- and 1.6-fold, respectively) in addition to reductions in the numbers of large muscle fibers (Gaworecki et al., 2012). Killifish also methylate and metabolize in very similar manner as humans do, making them a great model for arsenic toxicity (unpublished data).

The specific mechanism by which embryonic arsenic exposure reduces the overall size of animals and muscle fibers long after the exposure has ended is not well known. Therefore, we exposed killifish to 0, 50, 200 and 800 ppb \( \text{As}^{\text{III}} \) during embryogenesis, and performed cardiotoxin injections at 28, 40 and 52 weeks after rearing in clean water. We found that embryonic arsenic reduced the ability of killifish to recover completely after an induced skeletal muscle injury based on altered collagen and PCNA expression. These results indicate that embryonic-only arsenic may have permanent effects on muscle satellite cell function later on in life.
Methods

Killifish (*Fundulus heteroclitus*) collection and exposure to sodium arsenite

Adult killifish were collected using minnow traps from the Baruch Institute for Marine and Coastal Sciences in Georgetown, SC and maintained in the Aquatic Animal Research Laboratory at Clemson University. Fish were kept on a 16:8 light/dark cycle at 26°C in 18 ppt salt water (Coralife; Rhinelander, WI) and fed TetraMin flake food twice a day prior to egg collection. Embryonic exposure was performed as reported in Szymkowicz et al. 2017. Approximately 2,200 fertilized eggs were obtained, randomly separated into 28 Petri dishes, and exposed to 0, 50, 200 or 800 ppb sodium arsenite (equivalent to 0, 0.67, 2.67, or 10.67 μM; Fisher Scientific; Fair Lawn, NJ) in 18 ppt salt water: (n=7 Petri dishes per exposure). These concentrations represent exposures at the former drinking water standard (50 ppb) and arsenic levels in water as reported from a number of other countries that range from moderate to high levels (200 and 800 ppb) (Singh et al., 2015). Water in the exposure chambers was changed daily.

Within 24 hours of hatching, juvenile fish were removed from Petri dishes and placed in 38 liter tanks of arsenite-free 18 ppt salt water. Both the exposure and grow-out waters were maintained at a pH of 7.5-7.8, hardness of 180mg/L, and carbonate hardness of 120mg/L. Juvenile fish were fed brine shrimp for 4 weeks then gradually switched to TetraMin flake food, which contains a minimum protein content of 47%. Killifish were reared in clean water until they were sampled at 16, 28, 40 and 52 weeks of age. The time points were chosen to represent differing life and reproductive stages. At 16 weeks, killifish are still juveniles and cannot be sexed be examining outward physical characteristics, while at 28 weeks, coloration patterns in the males are just being established. By 40 weeks of age, eggs are being produced by the ovaries, but in the laboratory setting, are typically not released and fertilized. However, at 52 weeks,
reproduction can occur. After sampling, average condition factors (weight /length³) for each exposure group and sampling time were previously published (Szymkowicz et al., 2017). In general, condition factors increased as the fish aged, and were statistically significantly reduced at 16 and 28 weeks due to embryonic arsenic exposure.

*Cardiotoxin-induced skeletal muscle injury*

At each of the four sampling time points, three fish per tank (n=7 tanks per exposure group) were euthanized and trunk muscle between the tail and dorsal fin was removed for baseline measurements. At 28, 40 and 52 weeks, an additional two fish per tank were removed and lightly anesthetized using 0.1 g/L buffered MS-222. Cardiotoxin (Sigma, St. Louis, MO; 5 µL of a 10 mM solution in teleost saline) was injected into the trunk muscle just posterior to the dorsal fin. Cardiotoxin is a phospholipase found in snake venom and is used to examine skeletal muscle regeneration following an injury (Harris, 2003). After the injection, killifish were revived and maintained in a separate tank containing clean 18 ppt salt water and fed once daily. On days 3 and 10 post-injury, one fish per tank was euthanized and trunk muscle from dorsal fin to tail were collected to observe the progression of muscle recovery over 10 days.

*Collagen staining for muscle damage*

Muscle tissue samples were fixed in 10% neutral buffered formalin for 18 h, progressively dehydrated in ethanol, and embedded in paraffin. Blocks were cut into 7µm sections and slides were stained using Masson’s trichrome collagen staining kit (Richard-Allan Scientific; Kalamazoo, MI). Slides were imaged using a Leica DM750 light microscope and Leica EZ suite.
software. ImageJ software (National Institutes of Health) was used to quantify the amount of collagen present in the injury site. Using the circle tool, a 1.5 mm x 1.5 mm circle was placed over the injury site. After converting the image to an RGB (red, green, blue) stack and selecting the “red” channel, the threshold function was used to isolate the area of collagen. Percent area of collagen within the circle was then calculated using the “Measure” function. Average percent areas for day 3 and 10 post-injury samples were calculated for each exposure group (n=7) and baseline muscle samples (day 0) were calculated for each exposure group (n=3 per sex per tank) at 28, 40 and 52 weeks of age. Cardiotoxin injection was not done at 16 weeks due to the small size of the fish.

*Western Blotting for antibody validation*

Samples of trunk muscle and intestine were collected from control killifish to determine whether antibodies for PCNA and myogenin cross-reacted with killifish tissues. Tissue was homogenized in RIPA buffer containing protease inhibitors and was centrifuged at 10,000 rpm for 15 minutes at 4°C. Protein concentrations were determined using the Bradford assay (BioRad; Hercules, CA) and 30µg of protein was electrophoresed. PCNA (#sc-56; Santa Cruz Biotechnology, Dallas, TX) was used at 1:1000 dilution, myogenin (#F5D; Developmental Studies Hybridoma Bank, Iowa City, IA) was used at 1:750 dilution, and AP-labeled anti-mouse secondary antibodies were used at 1:2500. Blots were developed using BCIP and NitroBT and imaged with a BioRad gel imager.
**Immunohistochemistry to assess the number and localization of differentiating cells and myogenic precursors**

Formalin-fixed trunk muscle samples were also used for immunohistochemistry to quantify differentiating satellite cells (PCNA) and myoblasts (myogenin). Antigen retrieval was performed using a Tris-EDTA pH9 buffer followed by 3% hydrogen peroxide quench. PCNA (SCBT) was used at 1:400 dilution and myogenin (DSHB) was used at 1:100 dilution. Anti-mouse secondary antibody was used at 1:2000 dilution (VectaStain; Vector Labs, Burlingame, CA) and developed with NovaRED stain (Vector Labs). Myogenin-labeled samples were counterstained with 1X Gills hematoxylin for 1 minute. Baseline muscle samples and cardiotoxin injury samples were both imaged with a Leica DM750 light microscope and Leica EZ suite software. PCNA positive (+) nuclei were counted using ImageJ after converting the images to 16-bit and adjusting image contrast. Area of injury was also quantified using ImageJ trace tool. Average number of PCNA (+) cells were calculated for baseline samples (n=3 per sex per tank; 7 tanks per exposure) and for 3 and 10 day post-injury samples (n=5-7 tanks per exposure). To account for differences in the size of tissue injury, number of PCNA (+) cells was divided by the area of injury in the image field of view (40X; PCNA/mm²). Myogenin expression was quantified using the Color Deconvolution plugin for ImageJ. After selecting the injury area only, each image was separated into individual stain images (hematoxylin, NovaRED) using the H-DAB setting. Myogenin was then quantified using the threshold tool and adjusting the threshold value to be 140-180. Percent area and integrated density were determined for myogenin. Averages were calculated for baseline samples (n=3 per sex per tank; 7 tanks per exposure) and for 3 and 10 days post-injury samples (n=5-7 tanks per exposure). Cardiotoxin injection was not conducted at 16 weeks due to the small size of the fish.
Statistical analysis

Differences in baseline and cardiotoxin collagen, PCNA and myogenin expression were averaged across replicate tank (n=7) within each exposure group, and statistical differences assessed using one-way ANOVA and Tukey’s post-hoc test (p<0.05) with GraphPad Prism 4. Correlations between collagen, PCNA and myogenin were calculated using GraphPad Prism 4, and MANOVA contrasts were used to determine significant differences (p<0.05) between exposure groups at day 0, 3 and 10.

Results

Embryonic-only arsenic increases baseline collagen levels

Collagen staining of uninjured killifish trunk muscle was assessed to determine if baselines levels were affected by embryonic-only arsenic exposure (Fig. 1A-H). At 16 weeks, a dose-dependent increase in collagen expression was seen, with 227% greater levels in fish exposed to 800 ppb compared to controls (Fig. 1I). Fish were able to be sexed by 28 weeks. Since there were no significant differences in collagen expression between males and females (data not shown), all expression data was combined. Collagen expression at 28 weeks was significantly greater at 200 and 800 ppb, with 105 and 112% increases compared to the controls. While there were no significant changes at 40 weeks, collagen expression remained elevated at 52 weeks, where 200 ppb fish had 58% greater expression levels.
Embyronic-only arsenic delays collagen expression after skeletal muscle injury

To assess the ability to repair damaged skeletal muscle after injury, collagen expression was quantified at 3 and 10 days after cardiotoxin-induced injury. At 28 weeks of age (Fig. 2), collagen expression is decreased by 47% early in the repair process (day 3) in 800 ppb fish (Fig. 3A). Later in the repair process, at day 10, collagen expression is increased in both the 200 and 800 ppb exposed fish. However, none of the results are significant. At 40 weeks (Supplementary Figure 1), collagen expression reductions of 26-57% are seen in day 3 of the repair process in the 200 and 800 ppb exposure groups, respectively (Fig. 3B). By 52 weeks of age, collagen expression (Supplementary Figure 2) is slightly increased in the arsenic exposed groups at both day 3 and day 10 (Fig. 3C), with collagen expression being significantly elevated by 141% in 50 ppb fish.

Western blot for antibody validation

The reactivity of primary antibodies for PCNA and myogenin towards killifish skeletal muscle was determined through western blotting. Single bands for PCNA (36 kDa) and myogenin (40 kDa) were revealed at approximately the predicted molecular weights (Fig. 4). PCNA expression was found in both muscle and intestinal tissue (data not shown), further supporting its specificity to dividing cells. Myogenin was also seen at the appropriate size in the skeletal muscle tissue, but not in intestinal tissue (data not shown).
Embryonic-only arsenic increases baseline PCNA expression

These antibodies were then used to determine whether embryonic arsenic exposure altered their expression in killifish skeletal muscle. At 16 weeks, there is a dose-dependent increase in PCNA expression (Fig. 5A, B). Fish embryonically exposed to 800 ppb arsenic had 216% greater expression of PCNA compared to controls (Fig. 5I). Although no significant differences were seen at 28 weeks of age (Fig. 5C-D), the number of positive cells dose-dependently increases with higher arsenic exposures. At 40 weeks of age, all of the three exposure groups had between 94% and 187% significantly higher numbers of PCNA (+) cells (Fig. 5E-F, I). These elevated numbers persisted through 52 weeks, where 800 ppb fish had 48% more PCNA expression than controls (Fig. 5G-I).

PCNA levels are reduced in arsenic exposed fish following an injury

Proliferation of muscle satellite cells is the first step towards muscle repair and recovery after a skeletal muscle injury. Following a cardiotoxin injection into the trunk muscle of killifish, the number of PCNA (+) cells was assessed early on (3 days) and later (10 days) during the repair stages (Figure 6, supplementary Figures 3-4). At 28 weeks of age, PCNA levels are 39-41% lower in 200 and 800 ppb arsenic-exposed fish, respectively, at day 3 post-injury (Fig. 7A). At the later stage of repair on day 10, 200 and 800 ppb exposed fish still had between 40% and 60% lower PCNA expression compared to controls. At 40 weeks, while no significant differences in PCNA expression were seen at day 3 post-injury, at day 10 PCNA expression in both 200 and 800 ppb groups again is significantly reduced by 50-65%, respectively (Fig. 7B). At 52 weeks of age, similar trends in reduced PCNA expression remain. At day 3 post-injury, 50ppb exposed fish
have 55% less PCNA compared to controls, while at day 10, PCNA levels were significantly reduced by 74% in fish exposed to 200 ppb arsenic (Fig. 7C).

Embryonic-only arsenic exposure does not alter myogenin expression levels

Myogenin is a transcription factor expressed in myocytes that are differentiating to become myofibers, which are needed both during growth and after an injury. Following embryonic arsenic exposure and cardiotoxin injury, expression of myogenin was examined by immunohistochemistry. As the fish grow out between 16 to 52 weeks, there is a gradual increase in myogenin expression until the fish reach adulthood (Supp. Fig. 5A-I). However, there are no differences in myogenin expression between days or exposure groups (Supp. Fig. 6-9), except for 52 week old fish embryonically exposed to 200ppb on day 10.

PCNA, collagen, and myogenin correlations

Finally, we wanted to determine if there were any significant interactions between PCNA and collagen expression during the recovery period, so MANOVA contrasts were performed (Fig. 8). At 28 weeks, the 200 and 800 ppb groups were significantly different from controls at day 0, 3 and 10 (Fig. 8 A-C). While the response for day 0 is driven by higher PCNA and collagen in exposure groups compared to controls (Fig. 8A), at days 3 and 10 during the recovery period, the differences are driven by lower PCNA and higher collagen in exposed fish compared to controls (Fig. 8B-C). At 40 weeks, all three exposures are significantly different than controls at day 0 due to elevated PCNA levels (Fig. 8D). While there are no significant differences at day 3 (Fig. 8E), by day 10, the 200 and 800ppb groups are significantly different than the controls (Fig. 8F),
driven by reduced PCNA expression. At 52 weeks, the 50 and 200 ppb groups are significantly different than controls at day 0 due to higher collagen levels (Fig. 8 G). Only the 50 ppb group is different from controls at day 3 (Fig. 8H), and by day 10, all three exposure groups are different from the control group due to lower PCNA and higher collagen expression (Fig. 8I).

Comparisons were also made for PCNA and myogenin to look for any differences in proliferation (PCNA) and differentiation into myofibers (myogenin). At 28 weeks (Fig. 9 A), there were no differences between controls and exposed groups. Day 3 showed differences between 200 and 800 ppb groups and controls, mostly due to reduced PCNA (Fig. 9B). By day 10, the 200 and 800 ppb groups were still significantly different than controls (Fig. 9C). At 40 weeks, all three exposure groups were significantly different than controls on day 0 (Fig. 9D). Although there were no differences on day 3 (Fig. 9E), there was a dose-dependent trend seen on day 10, where 200 and 800 ppb groups had higher myogenin and lower PCNA expression (Fig. 9F). At 52 weeks, the 800 ppb group is significantly different from the control on day 0 (Fig. 9G). Day 3 shows an increase in myogenin and decrease in PCNA in exposed groups compared to the control, but only the 50 ppb group is different from controls (Fig. 9H). By day 10, the trend is reversed from day 3, where the control group has more PCNA and more myogenin present compared to exposed groups (Fig. 9I).

Collagen levels after injury and myogenin expression during the recovery period were also plotted for correlations. At 28, 40, and 52 weeks, several of the day 0 arsenic exposed groups were significantly different from controls because of increased collagen expression (Supp. Fig 10A, D, G). While there are no changes during the recovery period for week 28 or 40 fish, there is a decrease in both myogenin and collagen in the 52 week old fish on day 10 of the recovery period, with 50 and 200 ppb groups being significantly different than controls (Supp. Fig. 10I).
Discussion

The results from this study indicate that exposure to environmentally-relevant levels of arsenic during embryogenesis alters the normal development of skeletal muscle. However, following an injury to skeletal muscle, the early arsenic exposure impairs satellite cell proliferation and increases the amount of collagen deposited in the regeneration site.

Embryonic-only arsenic exposure alters the proliferation of muscle satellite cells

Arsenic is a developmental toxicant that can impair growth and weight gain during both embryonic and continuous exposures (Rahman et al., 2009; Kozul-Horvath et al., 2012; D’Amico et al., 2014; Kile et al., 2015). While earlier studies in killifish demonstrated a shift in skeletal muscle fiber sizes following exposure to high levels of arsenic (D’Amico et al., 2014), satellite cell proliferation has not been assessed. Because most cellular proliferation in adult fish skeletal muscle occurs when satellite cells respond to injury or environmental stressors (Johnston 2006), we used PCNA as a marker for their proliferation. The results of the current study indicate that the number of PCNA-positive muscle satellite cells was significantly reduced in response to an injury in killifish embryonically exposed to arsenic. Interestingly though, in the exposed, but uninjured fish, our results show a consistent dose-dependent increase in baseline PCNA expression from 16 to 40 weeks of age. The differences in PCNA levels persist through 52 weeks of age, although not to the same extent. The magnitude of PCNA expression at 16 weeks is much greater than at 28 weeks due to the initial growth of young fish. Although muscle growth in killifish continues through most of their lifetime, muscle production shifts from mosaic hyperplasia (increasing fast muscle fiber number) to hypertrophy of existing muscle fibers (Koumans and Akster, 1995; Johnston, 2006). Previous studies have shown that arsenic reduces
differentiation of embryonic stem cells by altering the expression of myogenic regulatory factors (Hong and Bain, 2012). Similar results were seen in C2C12 myoblasts, where arsenic exposure significantly impaired myotube formation (Yen et al., 2010). Further, PCNA protein expression in C2C12 myoblasts was significantly reduced after exposure to low-dose arsenic, indicating a reduction in proliferation (Liu et al., 2015). The results of all of these studies indicate that arsenic can impair skeletal muscle proliferation and differentiation.

Embryonic arsenic exposure has been shown to reduce the growth of killifish, with effects persisting out until at least 40 weeks of age (Szymkowicz et al., 2017). The current study revealed that as the fish grow out, the number of PCNA(+) cells in skeletal muscle are actually increased in a dose-dependent manner following embryonic arsenic exposure. We hypothesize that the elevated levels of PCNA expression throughout the lifetime of the fish may be a compensatory mechanism that allows organisms to catch up in growth (Szymkowicz et al., 2017).

Since the number of cells actively dividing in the muscle of arsenic-exposed fish was increased, we wanted to determine whether the same activation would occur during tissue repair and regeneration. Early in the repair process (day 3), muscle satellite cells are activated and begin proliferating to initiate the production of myoblasts (Dumont et al., 2015). Later in the repair process following proliferation, these myoblasts cease proliferation and begin to differentiate into myocytes and myofibers (Wang et al., 2014). Similar cardiotoxin injury and regeneration studies have been done in mice, where recovery and muscle regeneration was assessed between 2 and 14 days post-injury based on satellite cell activation (Yen et al., 2010; Mahdy et al., 2015; Hardy et al., 2016). Lower PCNA expression can be associated with reduced proliferation of muscle satellite cells, which would then lead to an impaired ability to recover after an injury (Liu et al., 2015; Dumont et al., 2015).
In addition to lower levels of PCNA in arsenic-exposed fish throughout the repair process, the initial increase of PCNA from day 0 to day 3 in immediate response to the injury is highly suppressed in exposed fish. Because of the dampened response of satellite cells, arsenic-exposed fish may not recover to the same extent as control fish do. Following skeletal muscle injuries in mice, decreases in tetanic force were seen up to 4 weeks post-injury in mice exposed to arsenic, in addition to reduced myogenicity of satellite cell myomatrices (Zhang et al., 2016). The dampening of the initial repair process continues as the fish age and can be seen during the recovery period in both 40 and 52 week old fish that were embryonically-exposed to arsenic. Thus, there appears to be long-term effects of embryonic-only arsenic exposure that prevents proper satellite cell differentiation and muscle repair after an injury.

*Embryonic-only arsenic exposure increases a fibrotic response following injury*

An alternative to the repair of injured tissue is to undergo fibrosis (Zhang et al., 2016; Karsdal et al., 2017). Since arsenic exposure altered satellite cell proliferation, we wanted to assess whether collagen levels were also changed. Embryonic arsenic exposure increased collagen expression in uninjured killifish muscle tissue at each time point examined.

After induction of an acute injury, damage to the basement membrane of epithelial or endothelial cells initiates the recruitment of a variety of inflammatory cell types and activation of muscle connective tissue fibroblasts and myofibroblasts (Midwood et al., 2004). Further, proteins such as thrombospondin 2, NF-kβ, osteopontin, and TGF-β that are typically associated with ECM deposition and pro-fibrotic responses are expressed (Zhang et al., 2016). The expression of these proteins leads to the deposition of collagen and other ECM proteins by fibroblasts and myofibroblasts. These signals also initiate the proliferation and differentiation in muscle satellite
cells to promote muscle regeneration (Tidball and Villalta, 2010). Collagen produced in skeletal muscle tissue during fibrosis could be collagen IV, laminin, or entactin (produced by epithelial cells and fibroblasts) or fibril-forming collagens I, III, V, and VI (produced by myofibroblasts), among other structural collagen fragments (Karsdal et al. 2017). Collagen IV is most commonly used for tissue repair processes and functions, whereas collagen I and III are more structural fibers. Collagen VI, on the other hand, is a major component in building the muscle extracellular matrix (ECM) and is crucial for the muscle satellite cell niche (Urciuolo et al., 2013; Cescon et al., 2015). Typically, collagen scar tissue is slowly remodeled after injury and is replaced by new muscle fibers. If the wound cannot heal properly or there is overactivation of fibroblasts, overproduction of ECM proteins can accumulate in the basement membrane (Karsdal et al., 2017). Low levels of collagen VI can lead to both impaired muscle regeneration and reduced ability of satellite cells to self-renew (Urciuolo et al., 2013). We used the Masson’s trichrome stain for collagen, however it does not differentiate between collagen types. During development, arsenic could have altered the ECM composition and led to a higher deposition of collagen and a more fibrotic state. These changes seem to be permanent, as the upregulated collagen is still present once the fish were adults.

As before, collagen levels in the injured fish were examined to assess its levels both early and late during the repair process. In general, collagen expression was increased in arsenic-exposed fish. This could be indicative of delayed healing or an overactivation of myofibroblasts, both of which would lead to higher levels of collagen. When correlating baseline PCNA and collagen levels at days 3 and 10 PCNA during muscle recovery, there are significant differences between control and exposed fish, which are mostly driven by higher collagen levels and lower PCNA levels. Because these differences, along with lower PCNA expression, remain consistent through
52 weeks of age, there may be a permanent change in the ability of muscle satellite cells to properly regenerate muscle tissue after an injury.

These results further suggest that arsenic induces a more fibrotic phenotype in skeletal muscle after embryonic-only exposures that persist into adulthood. Additionally, injury to skeletal muscle after arsenic exposures leads to an increase in collagen deposition at least 10 days after injury. Similar results have been reported in mice exposed to arsenic trioxide via drinking water for 8 weeks (Yen et al., 2010). In this study, mice treated with 0.5 and 5 ppm arsenic received glycerol injections in the soleus muscles to assess muscle regeneration in comparison to control mice. Arsenic-treated mice had much higher levels of collagen around the injury area compared to controls, indicating extensive fibrosis after arsenic treatment. In another study, mice were administered 100 ppb sodium arsenite via drinking water for 5 weeks and then a muscle injury was induced. The authors found that arsenic exposure reduced the number of new myofibers formed and increased alignment of collagen fibrils (Zhang et al., 2016). Another study reported the importance of collagen VI for proper renewal of muscle satellite cells (Urciuolo et al., 2013), where collagen VI-null mice had significantly fewer satellite cells after the late myogenic differentiation period 30 days after induced injury. Because arsenic has been shown to reduce differentiation of satellite cells and impair regeneration of new muscle fibers (Hong and Bain, 2012; Gaworecki et al., 2012; Ambrosio et al., 2014; Zhang et al., 2016), injured tissue could produce a more fibrotic phenotype instead of a myogenic recovery. Increased fibrosis due to arsenic exposure would then prevent proper healing and recovery of damaged tissue.
Conclusions

In summary, embryonic-only arsenic exposures alter proper muscle regeneration after injury up to one year of age, likely as a compensatory mechanism to allow the fish to catch up in growth. After a skeletal muscle injury, PCNA levels were reduced and collagen levels were increased through 52 weeks of age. Overall, these results suggest that muscle satellite cell function is a potential target of embryonic arsenic toxicity and a fibrotic recovery is favored.

Conflicts of interest

The authors declare that no conflicts of interest exist.

Acknowledgements

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References


**Figures**

Figure 3.1. Baseline collagen expression in muscle is increased following embryonic arsenic exposure. Sections of trunk muscle were formalin fixed, paraffin embedded, sectioned at 7µm, and stained with Masson’s trichrome. Collagen is stained blue (indicated by arrows), nuclei stained purple, and muscle fibers stained red. Representative images for 0 and 800 ppb fish for 16
weeks (A, B), 28 weeks (C, D), 40 weeks (E, F), and 52 weeks (G, H) are shown. Percent (%) collagen was quantified for 3 fish per tank per exposure (n=7) at 16 weeks, and 3 males and 3 females per tank per exposure (n=7) at 28, 40, and 52 weeks. Percent collagen was averaged across tank and exposure and presented for 16-52 weeks for males and females combined (I). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 3.2. Representative images of collagen expression in 28 week old killifish muscle after cardiotoxin injury. One fish per tank was immediately euthanized at 28 weeks of age (A, D), while two others were injected with cardiotoxin and allowed to recover until 3 days (B, E) or 10 days (C, F) post-injury. Tissue sections were stained with Masson’s trichrome for collagen expression. Expression of collagen was quantified using a 1.5mm x 1.5mm circle around the injury area (B-C, E-F).
Figure 3.3. Embryonic-only arsenic exposure increases collagen expression after injury.

Percent collagen was quantified and averaged across exposure group (n=3 per exposure) at week 28 (A), 40 (B), and 52 (C). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 3.4. Immunoblots of killifish PCNA and myogenin. Skeletal muscle protein (30µg) was electrophoresed bands were visualized through immunoblotting for myogenin (40kDa) and PCNA (36kDa).
Figure 3.5. Baseline PCNA expression in killifish trunk muscle is increased after embryonic arsenic exposure. Muscle tissue was formalin fixed, paraffin embedded, and sectioned at 7µm for immunohistochemistry. Tissue sections were incubated with PCNA and stained with NovaRED at 16 (A, B), 28 (C, D), 40 (E, F), and 52 weeks (G, H). The number of PCNA(+) cells
were counted for 3 fish per tank per exposure (n=7) at 16 weeks, and 3 males and 3 females per
tank per exposure (n=7) for 28-52 weeks. The total number of PCNA(+) cells was divided by the
area of injury (mm²) to determine PCNA(+)/mm², which was averaged across tank and exposure.
Data is presented for 16-52 weeks (I) for males and females combined. Statistical differences (*)
were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure 3.6. PCNA expression is reduced in skeletal muscle following a cardiotoxin injury. Two fish per tank were injected with cardiotoxin and allowed to recover for 3 or 10 days post-injury. Immunohistochemistry was used to examine levels of PCNA. Representative examples of PCNA expression in 28 week old fish are shown at 0 (A, D) 3 (B, E) or 10 (C, F) days post-injury.
Figure 3.7. Embryonic-only arsenic exposure reduces PCNA expression up to 10 days post-injury. Following immunohistochemical processing, the number of PCNA(+) cells were counted and averaged across exposures for day 0 (baseline), day 3, and day 10 post-injury for each exposure (n=3 per exposure) and divided by the injury area (mm$^2$) to determine PCNA/mm$^2$ at 28 (A), 40 (B), and 52 weeks (C). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
**Figure 3.8. Correlations between PCNA and collagen.** Average PCNA/mm$^2$ for each exposure was plotted against average percent (%) collagen for each exposure at 0, 3, and 10 days post-injury for 28 (A-C), 40 (D-F), and 52 (G-I) weeks of age (n=7 tanks per exposure). Statistical differences (*) were determined using MANOVA.
**Figure 3.9. Correlations between PCNA and myogenin.** Average PCNA/mm² for each exposure was plotted against average percent (%) myogenin for each exposure at 0, 3 and 10 days post-injury for 28 (A-C), 40 (D-F), and 52 (G-I) weeks of age (n=7 tanks per exposure). Statistical differences (*) were determined using MANOVA.
Figure S3.1. Representative images of collagen expression in killifish trunk muscle after cardiotoxin injury at 40 weeks. Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 (C, F) days post-injury, where one fish was sampled at each time point. Day 0 indicates baseline collagen (A, D). Tissue sections were stained with Masson’s trichrome for collagen expression. Expression of collagen was quantified using a 1.5mm x 1.5mm circle around the injury area.
Figure S3.2. Representative images of collagen expression in killifish trunk muscle after cardiotoxin injury at 52 weeks. Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 (C, F) days post-injury, where one fish was sampled at each time point. Day 0 indicates baseline collagen (A, D). Tissue sections were stained with Masson’s trichrome for collagen expression. Expression of collagen was quantified using a 1.5mm x 1.5mm circle around the injury area.
Figure S3.3. PCNA expression in killifish trunk muscle after cardiotoxin injury at 40 weeks.

Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 (C, F) days post-injury, where one was sampled at each time point to assess recovery and function of muscle satellite cells. Day 0 indicates baseline PCNA levels (A, D). PCNA expression was examined by immunohistochemistry using NovaRED stain.
Figure S3.4. PCNA expression in killifish trunk muscle after cardiotoxin injury at 52 weeks.

Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 (C, F) days post-injury, where one was sampled at each time point to assess recovery and function of muscle satellite cells. Day 0 indicates baseline PCNA levels (A, D). PCNA expression was examined by immunohistochemistry using NovaRED stain.
Figure S3.5. Baseline myogenin expression in killifish trunk muscle. Muscle tissue was formalin fixed, paraffin embedded, and sectioned at 7µm for immunohistochemistry. Tissue sections were incubated with myogenin and stained with NovaRED at 16 (A, B), 28 (C, D), 40 (E, F), and 52 weeks (G, H). Percent (%) myogenin was quantified using ImageJ for 3 fish per
tank per exposure (n=7) at 16 weeks, and 3 males/females per tank per exposure (n=7) for 28-52 weeks and was averaged across tank and exposure. Data is presented for 16-52 weeks (I) for males and females combined. Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
Figure S3.6. Representative images of myogenin expression in killifish trunk muscle after cardiotoxin injury at 28 weeks. Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 days (C, F) post-injury, where one fish was sampled at each time point. Day 0 indicates baseline myogenin levels (A, D). Muscle tissue was used for immunohistochemical analysis of myogenin and stained with NovaRed.
Figure S3.7. **Representative images of myogenin expression in killifish trunk muscle after cardiotoxin injury at 40 weeks.** Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 days (C, F) post-injury, where one fish was sampled at each time point. Day 0 indicates baseline myogenin levels (A, D). Muscle tissue was used for immunohistochemical analysis of myogenin and stained with NovaRed.
Figure S3.8. Representative images of myogenin expression in killifish trunk muscle after cardiotoxin injury at 28 weeks. Three fish per tank were injected with cardiotoxin and allowed to recover until 3 (B, E) or 10 days (C, F) post-injury, where one fish was sampled at each time point. Day 0 indicates baseline myogenin levels (A, D). Muscle tissue was used for immunohistochemical analysis of myogenin and stained with NovaRed.
Figure S3.9. Embryonic-only arsenic exposure does not alter myogenin expression 10 days post-injury. Following immunohistochemical processing, the injury area was outlined using ImageJ and percent myogenin was quantified using the color deconvolution plug-in. Percent myogenin was averaged across exposures for day 0 (baseline), day 3, and day 10 post-injury for each exposure (n=3) at 28 (A), 40 (B), and 52 weeks (C). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p<0.05).
**Figure S3.10. Correlations between collagen and myogenin.** Average percent collagen for each exposure (n=3) at each time point was plotted against average percent (%) myogenin for each exposure (n=3) at 0, 3 and 10 days post-injury for 28 (A-C), 40 (D-F), and 52 (G-I) weeks of age. Statistical differences (*) were determined using MANOVA.
CHAPTER FOUR

EMBRYONIC ARSENIC IMPAIRS OLFACTORY SENSORY NEURON DIFFERENTIATION AND FUNCTION INTO ADULTHOOD IN KILLIFISH (FUNDULUS HETEROCPLITUS).

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Abstract

Arsenic is a common worldwide contaminant of food and drinking water. Epidemiological studies have reported correlations between arsenic exposure and neurodevelopmental abnormalities such as lower IQ, reduced memory and sensory functioning, while in vitro studies have shown that arsenic reduces neurogenesis and alters stem cell functions. The goal of this study was to use killifish (Fundulus heteroclitus) as a model organism to determine if embryonic-only arsenic exposure alters olfactory stem cell numbers and/or functions, and if so, whether those changes persist into adulthood. Killifish embryos were exposed to 0, 10, 50 or 200 ppb arsenite (As\textsubscript{III}) until hatching, and juvenile fish were raised in clean water. At 0, 2, 4, 8, 16, 28 and 40 weeks of age, behavior tests were performed to assess start time and percent response in reaction to various odorants that activate specific olfactory sensory neurons, including taurocholic acid (ciliated cells), amino acids (microvillus cells), and female pheromones (crypt cells). Olfactory epithelia were then collected for immunohistochemical analysis of stem cell populations (PCNA/Sox2), as well as the expression of ciliated cells (calretinin), and microvillus cells (G\textsubscript{αi3}) at 0, 4, 16 and 28 weeks. In response to pheromones, there was a significant reduction in percent response of 39-47\% and 68-85\% increase in start time at week 0 in exposed fish, and these reductions persisted through 28 weeks. In response to TCA, start time was initially increased by 68-72\% in exposed fish at week 4 and percent response was reduced by 39-42\% through week 28. In response to amino acids, start time was increased by 188-302\% at week 4 in exposed fish. By week 28, start time was increased by 112\% and percent response was reduced by 55\%. When specific cell types were assessed by immunohistochemistry, cells undergoing proliferation were reduced until week 16, and at week 28 there is a 43-68\% increase in PCNA expression. Sox2 expression is decreased in exposed fish by approximately 35\% until week 28, where no differences persist. Calretinin expression is consistently reduced in exposed fish from
week 4 to week 28, about 24-27% lower than controls. $G_{\alpha3}$ expression remained unaffected by arsenic exposure until week 16, where it was reduced by 32-39%. Based on these results, embryonic-only exposure to arsenic seems to have long-term effects on behavior due to altered development of the olfactory epithelium in killifish. These changes in proliferation and differentiation of neural stem cells during growth may have detrimental effects on adult neurogenesis.

**Key words:** arsenic, olfactory sensory neurons, killifish, PCNA, Sox2, stem cells
Introduction

Arsenic (As) is a naturally occurring metalloid found throughout the world in the air, water and soil. Millions of people are exposed to arsenic daily through contaminated drinking water and food sources such as rice, fish and apple juice. The current drinking water standard set by the World Health Organization is 10 parts per billion (ppb), or 10 µg/L. However, some countries have standards set at 50 ppb, and many groundwater sources and private wells exceed these regulations (Mandal & Suzuki, 2002; Naujokas & Anderson, 2013; Ng, Wang, & Shraim, 2003). Chronic arsenic exposure is known to cause kidney, liver, skin and bladder cancer, in addition to respiratory and cardiovascular diseases. The goal of these drinking water standards is to protect against carcinogenic effects, but it is unknown if they are also protective of non-carcinogenic effects of arsenic.

Many studies have established a correlation between neurologic deficits and arsenic exposure. Low level arsenic exposure in adults has produced sensory neuropathies and abnormal sensory nerve function, in addition to irritability, depression, sleep disorders (Mukherjee et al., 2003). Further epidemiological studies link arsenic exposure to decreased intelligence quotients, long-term memory, and neuropsychological changes (Kapaj, Peterson, Liber, & Bhattacharya, 2006; Tolins, Ruchirawat, & Landrigan, 2014; Yorifuji et al., 2016). Arsenic is known to cross both the placenta and the blood-brain barrier (BBB), which poses significant concern for fetuses that are exposed through maternal drinking water (Tolins et al., 2014). Exposure to arsenic through maternal intake produced significant reductions in verbal and full-scale IQ in female children up to 5 years of age in both prenatal to early life and concurrent exposures (Hamadani et al., 2011). Negative associations were also seen between urinary arsenic (<50 ppb) and visual-spatial tests, picture recognition, and problem-solving tests in children 6-8 years of age (Rosado et al., 2007). Similar neurobehavioral changes and memory deficits have been seen in prenatal
exposures in rodents. Significant delays in righting reflexes, cliff aversions, and negative geotaxis were seen in female rats exposed to 50 and 100 ppb arsenic in maternal drinking water (Gumilar, Lencinas, Bras, Giannuzzi, & Minetti, 2015).

Many studies have reported the impacts of arsenic exposure on the proliferation, differentiation, and function of multiple stem cell types. For example, arsenic exposures as low as 7.5 ppb significantly inhibited differentiation of mouse embryonic stem cells into neurons (Hong & Bain, 2012), and human neural stem cells exposed to 2-4 µM arsenite showed significantly decreases in Nanog expression required for pluripotency of stem cells in addition to suppression of the PI3K-AKT pathway (Ivanov & Hei, 2013). Further, exposure to 50 ppb arsenite in maternal drinking water significantly delayed differentiation of embryonic mouse neural progenitor cells through the REST/NRSF pathway (Tyler, Labrecque, Solomon, Guo, & Allan, 2017).

Proper number and function of neural stem cells is essential for the development and maintenance of the olfactory system. Neurons involved in olfaction are produced by neural stem cells both during development and throughout adulthood, making the olfactory system an ideal model for adult neurogenesis (Lindsey, Di Donato, Kaslin, & Tropepe, 2014). The olfactory system is a complex network of pathways and signals that is highly conserved across vertebrates. In fish, the olfactory system is crucial for various sensory behaviors needed for reproduction, feeding, socialization, and predator avoidance. Olfaction occurs through activation of G-protein coupled receptors (GPCRs) on olfactory sensory neurons (OSNs), which are passed through the olfactory nerve and into the olfactory bulb and sent to the telencephalon for processing (Bazáes, Olivares, & Schmachtenberg, 2013; Hino, Miles, Bandoh, & Ueda, 2009). The olfactory epithelium has multiple cell types that are specific to certain odorants: ciliated OSNs respond to bile salts and steroids, microvillus OSNs respond to amino acids and food cues, and in fish, crypt
OSNs are thought to respond to pheromones (Kaupp, 2010). The olfactory epithelium is extremely unique in that neurogenesis occurs throughout adulthood through basal stem cells and progenitor cells. If these stem cell populations are reduced or functionally impaired, both neurodevelopment and adult neurogenesis could be altered and ultimately lead to sensory dysfunction.

Killifish (*Fundulus heteroclitus*) are a commonly used animal model for developmental studies (Burnett et al., 2007). They produce large, transparent eggs that are fully developed in 14-21 days in laboratory settings. Compared to rodent models, they produce a large number of offspring and offer greater statistical power. Killifish metabolize arsenic also in a manner more similar to humans compared to rodent models, which have differences in arsenic methylation efficiency (Rehman & Naranmandura, 2012). Previous studies have exposed killifish to 0-800 ppb arsenic embryonically and reported significant alterations in swimming behavior, location in tanks, and response to food stimulus (Szymkowicz, Sims, Castro, Bridges, & Bain, 2017).

The specific mechanism behind altered sensory neuronal function and changes in behavior after arsenic exposures are not well known. In the current study, we exposed killifish embryos to environmentally relevant concentrations of arsenic during embryogenesis only and raised the hatchlings in clean water for up to 40 weeks. Odorant responses and olfactory epithelial cell subtype populations were examined to determine if arsenic altered sensory responses or neural stem cell populations. We found that embryonic arsenic altered responses to bile salts and pheromones well into adulthood, which was accompanied by reductions in neural stem cell populations in the olfactory epithelium.
Methods

*Killifish (Fundulus heteroclitus) collection and sodium arsenite exposure*

Adult male and female killifish were collected from the Baruch Institute for Marine and Coastal Ecology at the North Inlet National Estuarine Research Reserve (NERR) in Georgetown, SC and maintained in the Aquatic Animal Research Laboratory at Clemson University. Adult killifish were housed in 18 ppt salt water (Coralife; Rhinelander, WI) on a 16:8 light/dark cycle at 26°C. Fish were fed zebrafish pellet food (Zeigler Brothers; Gardners, PA) twice a day prior to egg collection and fertilization.

To observe early olfactory injury due to embryonic-only arsenic, eggs and milt were collected from male and female adult fish and mixed together for fertilization. Approximately 2,200 fertilized eggs were randomly separated into 24 Petri dishes. Embryos were exposed to 0, 10, 50 or 200 ppb sodium arsenite (n=6 dishes per group; 40 eggs per dish) (Fisher Scientific; Fair Lawn, NJ). Killifish were removed from the arsenite exposure within 24 hours of hatching and placed into 3.7 L zebrafish tanks with arsenite-free 18 ppt salt water (Coralife; Rhinelander, WI). Juvenile fish were fed brine shrimp twice a day. Odorant responses tests were conducted, and killifish were sacrificed (n=3 per tank) at 0, 2, and 4 weeks post-hatch for olfactory epithelia collection.

To observe long-term effects of embryonic-only arsenic exposure, and to avoid overcrowding, a second set of fertilized eggs were collected and randomly dispersed into 28 Petri dishes. As with the early time point study, embryos were exposed to 0, 10, 50 or 200 ppb sodium arsenite (n=7 dishes per exposure group; approximately 90 eggs per dish) (Fisher Scientific; Fair Lawn, NJ). Within 24 hours of hatching, juvenile fish were removed from the Petri dishes and placed into 10 gallon tanks of arsenite-free 18 ppt salt water. Juvenile killifish were maintained
on a brine shrimp diet until 4 weeks of age then gradually switched to Zeigler adult zebrafish diet (Gardners, PA). Zeigler food was analyzed for inorganic arsenic content and non-detectable levels were reported (data not shown). Odorant response tests were conducted, and killifish were sacrificed (n=3 per tank) at 8, 16, 28 and 40 weeks of age for olfactory epithelia collection.

**Odorant behavioral tests**

To look for initial injury to olfactory sensory neurons (OSNs) and their ability to respond to odorants, behavior tests in response to various odorants were performed. At week 0, hatchlings were removed from the exposure Petri dishes and placed into 3.7 L zebrafish tanks. After 70% of the embryos hatched, the test was initiated. Behavior was initially recorded for 20 seconds using a Canon video camera to capture “normal” behavior. Then, 1 mL of a 250 mM taurocholic acid (TCA) solution was added into the top of the water column in each tank to induce a socialization response, and fish behavior was video recorded for an additional 60 seconds. Two additional behavior tests were conducted over the next 24 hours using 1 mL of an amino acid mixture (250 mM each of L-methionine, L-histidine, L-cysteine, L-valine, L-alanine) to induce a feeding response, and with 0.4 mL of killifish pheromone extract (1.5g female egg sacs homogenized in 30 mL phosphate-buffered saline) to induce a mating response. At weeks 2 and 4, behavior tests were performed once every 8-10 hours in the 24-hour time period before euthanasia. Videos were analyzed for time to start, which was determined as the time when 10% of the total fish started responding to the odorant, and percent response of killifish.

To look for long-term changes in olfactory function, the same odorant response tests were also performed at 8, 16, 28 and 40 weeks of age. Three weeks prior to each destructive sampling time point, killifish were exposed to one of the three different odorants each week. Behavior tests
for each odorant were repeated twice within the week. Functioning of microvillus OSNs were assessed after addition of 4 mL of a mixture of 250 mM amino acids to the top of the water column. Functioning of ciliated OSNs were observed with addition of 4 mL of 250 mM TCA, and that of crypt OSNs were determined using 1 mL of killifish ovarian extract. Killifish behavior was recorded for 20 seconds before addition of each odorant to observe “normal” behavior. After the odorant addition, behavior was video recorded for an additional 60 seconds for amino acids and pheromones, and 120 seconds for TCA. Videos were analyzed for time to start and percent response of killifish.

**Western blotting for antibody validation**

Control adult killifish were euthanized using buffered MS-222 and samples of olfactory epithelia were collected for protein extraction. Immunoblotting was used to determine whether antibodies for PCNA, Sox2, calretinin, and $G_{\alpha i3}$ cross-reacted in killifish olfactory epithelial tissue. Tissues were homogenized in RIPA buffer with protease inhibitors and were centrifuged at 10,000 rpm for 15 minutes at 4 °C. Protein concentrations were determined using the Bradford assay (BioRad; Hercules, CA) and 30 µg of protein was electrophoresed onto SDS polyacrylamide gels. Following transfer to nitrocellulose, primary antibodies for PCNA (#sc-56; Santa Cruz Biotechnology, Dallas, TX), Sox2 (ab97959; Abcam), calretinin (ab702; Abcam), and $G_{\alpha i3}$ (sc365422; Santa Cruz Biotechnology, Dallas, TX) were each used at 1:1000 dilution. AP-labeled anti-mouse and anti-rabbit secondary antibodies were used at 1:2500 dilution. Blots were developed using NitroBT and BCIP and imaged with a BioRad gel imager.
Immunohistochemical analysis of olfactory epithelia

To assess changes in numbers and localization of specific OSNs, stem cells, and proliferating cells in the olfactory epithelia, three fish per tank were euthanized in 1 g/L buffered MS-222 at weeks 0, 4, 16, and 28 and olfactory epithelia were collected. Tissue was fixed overnight in 10% neutral-buffered formalin, then processed and embedded in paraffin. Olfactory epithelia were cut into 5 µm sections. Antibodies for proliferating cells (PCNA; sc-56), neural stem cells (Sox2; ab97959), ciliated cells (calretinin; ab702), and microvillus cells (Gaβ3; sc-365422) were used to quantify each cell type. Antigen retrieval was performed using a sodium citrate pH6 buffer (PCNA, Sox2, Gaβ3) and a Tris-EDTA pH9 buffer (calretinin). Samples were blocked overnight at 4 °C in 5% BSA, 10% goat serum, and 0.1% Tween 20. Primary antibodies were used at 1:100 dilution and incubated overnight at 4°C. Alexa Fluor 488 anti-mouse and Alexa Fluor 532 anti-rabbit secondary antibodies were used at 1:200 dilution for 1hr at room temperature. All samples were counterstained with DAPI. Samples stained with PCNA or Sox2 were imaged using a Leica SPE confocal microscope, equipped with traditional PMT detectors and a 63X objective. For PCNA, Sox2, and DAPI, excitation wavelengths were 488, 532, and 406 nm, respectively, and emission wavelengths were 500-560, 535-600, and 430-480 nm, respectively. Samples stained with calretinin and Gaβ3 were imaged using a Leica SP8X confocal microscope, equipped with HyD detectors, time gating, a 63X objective (N.A= 1.4) with 1.5 zoom, and a tunable laser. An excitation wavelength of 532 nm with emission wavelengths of 540-595 nm were used for calretinin, and excitation wavelength of 495 nm with emission wavelengths of 498-560 nm was used for Gaβ3. Imaging conditions were held consistent for all samples from each time point of development to allow for comparisons. Images were analyzed using ImageJ (NIH). For PCNA and Sox2, the number of cells expressing each antigen was counted (n = 4-6). For calretinin and Gaβ3, average integrated density was determined (n = 4-6).
**Statistical analysis**

Differences in start time, duration and percent response were averaged across replicates \((n=7)\) for each exposure group. Number of PCNA and Sox2 cells were also averaged across replicates \((n=4-6)\) for each exposure group at each time point. Integrated density of calretinin and \(G_{\alpha3}\) were converted to fold change of week 0 control and averaged across replicates \((n=4-6)\). Statistical differences for each parameter were determined using one-way ANOVA and Tukey’s post-hoc test \((p < 0.05)\) with JMP Pro 12. Correlations between start time and percent response for each odorant with PCNA, Sox2, calretinin, and \(G_{\alpha3}\) expression were calculated using JMP Pro 12, and MANOVA contrasts were used to determine significant differences between exposure groups \((p < 0.05)\).

**Results**

*Embryonic-only arsenic alters behavioral responses to pheromone stimulus*

Behavior tests to assess responses to various odorants were used to determine if embryonic arsenic exposure altered the development and function of olfactory sensory neurons, and if these changes persisted into adulthood. Killifish pheromone extracts were used as an odorant to activate sensory neurons in crypt cells. At week 0, there was a significant dose-dependent increase in start time, where exposed killifish take 68-85% longer to respond (Fig. 1A). At week 2, only the 200ppb exposed fish take significantly longer to respond to the pheromones, and by week 4, there are no longer significant increases in start time (Fig. 1A). However, as sexual maturity progresses, the 10 and 50ppb groups have a significant 56-60% increase in start time by 8 weeks (Fig. 1A). At weeks 16 and 28, all three exposure groups have significantly greater start times, and this increase in time to start remains at 40 weeks of age (Fig. 1A). The patterns in the
percentage of fish responding to pheromones is concordant to the delayed start times, in that at week 0, percent response is also significantly reduced by 39-47% in exposed killifish (Fig. 1B) and this reduction remains for the 200 ppb exposure group until week 4. At weeks 16 and 28, percent response is significantly reduced in most of the exposure group by 32-52% (Fig. 1B).

*Embryonic-only arsenic alters socialization responses following a TCA stimulus*

Taurocholic acid (TCA) is a bile salt that is commonly used to initiate socialization responses in fish, and exposure to this odorant typically activates ciliated cells in the olfactory epithelium. At week 0, there were no significant differences in start time or percent response. At week 2, start time was significantly increased by 52% in 200ppb fish, while by week 4, start time was significantly increased in 50 and 200ppb fish by 68-72% (Fig. 2A). While trends exist for delayed start time in the remaining weeks, only week 28 fish were statistically different from controls (Fig. 2A). In addition to alterations in start time, the percentage of fish responding to TCA was significantly impaired, with 39-55% less fish responding at week 2, and 39-42% less fish responding at week 28 (Fig. 2B). By week 40, there continued to be a dose-dependent trend in percent response, with a 32% reduction in 200ppb fish (Fig. 2B).

*Embryonic-only arsenic alters behavioral responses to amino acids*

To assess activation of microvillus cells in the olfactory epithelium, an amino acid mixture was administered to the water column of each tank to induce a feeding response. At week 0, there were no differences in start time in any exposure group. At week 2, start time was significantly increased by 131-139% in 50 and 200ppb fish, and this delay remained in week 4 fish, whose time to start was significantly increased by 188-302% in 50 and 200 ppb fish (Fig. 3A). After
these earlier changes, there is either no or minor changes in start time as the fish age. In a similar fashion, percentage of fish responding to TCA is impaired early on, with reductions in fish exposed to 200 ppb arsenic at weeks 0 and 2 (Fig. 3B). No other changes in percent response were noted as the fish age.

*Embryonic-only arsenic alters PCNA and Sox2 expression in killifish*

Immunohistochemistry was used to assess stem cell populations in the olfactory epithelium at 0, 4, 16 and 28 weeks of age in order to determine if arsenic affects proliferation (PCNA) and populations of neural stem cells (Sox2) both immediately after exposure and later in life (Fig. 4-5; Supp. Fig. 1,2). At week 0, PCNA expression is reduced by 40% and Sox2 expression is reduced by 35% in fish exposed to 50 ppb arsenic (Fig. 6A, B). At week 4, PCNA expression is reduced by 42-52% in 10 and 200 ppb groups, respectively, and Sox2 expression is decreased by 20% in 200 ppb fish, although not statistically significant. However, at week 16 PCNA expression is increased by 65% in 200 ppb fish and Sox2 expression remains suppressed by 23-34% (Fig. 6A, B). By week 28, PCNA expression remains elevated by 43-68% in 50 and 200 ppb fish. Sox2 expression is no longer affected by arsenic exposure at week 28 (Fig. 6B).

*Embryonic-only arsenic alters calretinin and Gαi3 expression*

Immunohistochemistry was also used to determine if embryonic arsenic exposure affected the presence of specific olfactory cell types in the epithelium. Multiple antibodies were tried to assess the presence of crypt cells, but none reacted with killifish epitopes. Therefore, expression of calretinin (ciliated cells) and Gαi3 (microvillus cells) were analyzed (Fig. 4-5; Supp. Fig. 1,2). In order to compare changes in expression as the fish aged, calretinin expression was normalized to
week 0 controls. $G_{\alpha i3}$ expression was compared to the control at each time point due to variable microscope laser settings. When comparing calretinin at week 0 to week 28 in controls, we see a gradual, delayed increase in expression due to the proliferation and differentiation of neural stem cells into neuroblasts, which is eventually followed by further differentiation into specific olfactory sensory neurons (ciliated and microvillus cells). At week 0, calretinin expression was increased by 72% in 50 ppb fish while $G_{\alpha i3}$ expression was not changed (Fig. 7A, B). At week 4, there is a dose-dependent decrease in calretinin expression, though not significant (Fig. 7A). There was a slight increasing trend in $G_{\alpha i3}$ expression but was not significant (Fig. 7B). At week 16, the decreasing trend of calretinin expression with arsenic continues, and $G_{\alpha i3}$ switched to a decreasing trend, with 10 and 50 ppb groups being reduced by 37-42% (Fig. 7A, B). By week 28, calretinin expression continues to be suppressed by 119-157% (Fig. 7A). A slight decreasing trend in $G_{\alpha i3}$ expression continued through week 28 but was not significant (Fig. 7B).

Correlations between IHC and odorant responses

Following analysis of both behavioral responses and olfactory epithelium IHC, we wanted to look for correlations between specific cell types and their functions as displayed by differences in behavior. To determine if any correlations were significant, we performed MANOVA contrasts (Fig. 8). Correlations between PCNA expression (proliferation) and pheromone response were first examined. At week 0, all exposure groups are significantly different from the control group, driven by reductions in both percent response to pheromones and PCNA expression (Fig. 8A). At week 4, the 200ppb group is still different than the control due to lower levels of PCNA (Fig. 8B). By week 16, however, PCNA expression increases in the 200ppb group even though response to pheromones is still lower than the controls (Fig. 8C). By week 28, all exposed groups are significantly different than the controls, which have higher expression of PCNA coupled with
reductions in response to pheromones (Fig. 8D). When comparing Sox2 expression (neural stem cells) with response to pheromones, the 50 and 200ppb groups are significantly different than the controls at week 0 due to both lower Sox2 levels and reduced percent response (Fig. 8E). At week 4, there are no significant differences between control and exposure groups, although there remains a decreasing trend in percent response (Fig. 8F). By week 16, all three exposure groups are significantly different than the control due to higher Sox2 levels and greater responses in the controls (Fig. 8G). Finally, at week 28 there seems to be a recovery in Sox2 expression but reductions in percent response persist (Fig. 8H).

Next, we performed MANOVA contrasts with TCA response and both PCNA and Sox2 expression (Fig. 9). At week 0, lower PCNA levels drive the differences between the 50 and 200ppb groups and the control group (Fig. 9A). At week 4, the 10 and 200ppb groups are significantly different than the controls, driven by the reduced PCNA expression in both groups (Fig. 9B). By week 16, however, PCNA levels are higher in the 200ppb group compared to the control while there are no differences in response to TCA. This also continues through week 28, with all groups having higher levels of PCNA than the control (Fig. 9D). Significant differences are driven by both PCNA levels and reductions in percent response to TCA. Regarding Sox2 expression compared to percent response to TCA, similar trends to PCNA expression are seen, with the 50ppb group being significantly different from the control due to lower Sox2 expression (Fig. 9E). At week 4, there are no differences between groups (Fig. 9F). At week 16, however, all exposure groups are significantly different than the control group due to lower Sox2 expression and reduced response (50 and 200ppb) (Fig. 9G). By week 28, the 50 and 200ppb groups are significantly different than the control only due to reductions in response to bile salts (Fig. 9H).

Finally, we compared response to TCA with the expression of calretinin, a marker for ciliated OSNs that respond to bile salts (Fig. 10). At week 0, the 50ppb group is significantly different
than the control due to elevated levels of calretinin (Fig. 10A). At week 4, a dose-dependent trend is seen with reductions in both percent response to TCA and calretinin expression, with the 200ppb group being significantly different (Fig. 10B). At week 16, the 50 and 200pb groups are significantly different from the control due to reductions in response to TCA (Fig. 10C). By week 28, all three groups are significantly different from the control group due to both reductions in calretinin expression and percent response to TCA (Fig. 10D).

Correlations between IHC markers and start time for each odorant were very similar to the correlations with percent response (data not shown). MANOVA contrasts were also performed for response to amino acids and levels of PCNA or Sox2 expression (Supp. Fig. 3). At week 0, all three groups are different from the control due to PCNA levels (Supp. Fig. 3A). These differences remain at week 28 (Supp. Fig. 3D). For Sox2, all groups are different than the control at week 16 (Supp. Fig. 3G). Correlations were also performed for comparisons between response to amino acids and G_{\alpha i3} expression, the marker for microvillus cells that respond to amino acid stimuli (Supp. Fig. 4). There were no differences between groups until week 16, where the 10 and 50ppb groups had lower G_{\alpha i3} expression (Supp. Fig. 4C).

Discussion

The results from this study indicate that embryonic-only exposure to environmentally-relevant levels of arsenic alters the proliferation and differentiation of neural stem cells in the olfactory epithelium, as seen by changes in PCNA and Sox2 expression lasting through week 28 post-exposure. The impairment in turn produced changes in killifish behaviors in response to various odorants that persisted well into adulthood.
Embyronic-only arsenic exposure alters behavior in response to various odorants

Arsenic is a well-known developmental toxicant that has been shown to alter behaviors associated with neurodevelopment. In zebrafish, exposures to 1-100 ppb arsenic impaired behavioral responses required for assessing memory (de Castro et al., 2009). Rodent studies have also shown learning deficits and impaired sensory-motor behaviors after prenatal exposures (Gumilar et al., 2015; Luo et al., 2013). While many studies such as these have investigated the effects of arsenic on memory, learning, and intelligence, few have focused on the impact on the developing olfactory system and sensory function. OSNs in the olfactory epithelium are constantly exposed to compounds and contaminants from the external environment. Because of this, OSNs undergo constant death and renewal throughout a lifetime, making the olfactory system an ideal model for adult neurogenesis.

Previous studies using killifish have reported a reduced response to a food stimulus following embryonic arsenic exposure (Szymkowicz et al., 2017). In order to remove the visual stimulus and to target the function of all three cell types of olfactory sensory neurons, we administered solutions of female adult pheromones, taurocholic acid (TCA), and an amino acid mixture to activate crypt cells, ciliated cells, and microvillus cells, respectively. The results of this study indicate that embryonic-only arsenic exposures increased the time it took for fish to respond to each of the odorants and decreased the number or percentage of fish that responded to the odorants, with some of these effects persisting through the entire 40 week length of the study. Immediately after hatching, the time it took to start responding was significantly longer after the addition of pheromones. Similar trends were seen with TCA and amino acids, but not to the same extent. By week 4 post-exposure, significant increases in start time were seen in response to TCA and amino acids. Start time continued to be increased through week 40 in response to pheromones in fish exposed to as low as 10ppb arsenic, indicating long-term olfaction impairments with little
recovery. In contrast, differences in start time for TCA ceased after week 28, and ended by week 8 for amino acids, suggesting a recovery of the ciliated and microvillus cells. Very similar trends are present for percent response of fish towards the odorants.

Pheromones, TCA, and amino acids have been commonly used in previous fish behavioral studies as stimulators for the three olfactory sensory neuron subtypes found in the olfactory epithelium. For example, one study used L-amino acid mixtures and TCA in olfactory studies to determine functional properties of microvillus and ciliated cells in channel catfish (Hansen et al., 2003). After brief exposures to cadmium or zinc, other studies have reported decreases in response to TCA and L-cysteine in zebrafish (Heffern, Tierney, & Gallagher, 2018; Matz & Krone, 2007). Further, chemical lesioning of the olfactory epithelium with Triton X-100 led to reductions in responses to bile salts in zebrafish but not to amino acids (White, Kounelis, & Byrd-Jacobs, 2015). Salmon that were exposed to waterborne cadmium exhibited behavioral changes in response to L-cysteine that persisted after a 16-day depuration period (Williams et al., 2016). Immunostaining revealed that although there were no changes in Sox2 expression, expression of adenylate cyclase III, a signal transduction protein found only in ciliated OSNs, was severely reduced. Copper exposures to salmon have produced similar changes in expression of olfactory sensory genes, specifically those present in ciliated cells (Wang, Espinoza, & Gallagher, 2013).

Our data indicates that embryonic arsenic exposure can also impair responses to TCA, although there is an eventual recovery in adulthood. In addition, our study also demonstrated that pheromone response was permanently reduced in killifish exposed to arsenic, which is believed to be indicative of a loss of crypt cell function. Indeed, there are conflicting results as to the true function and activation of crypt cells. One study found that a small population of crypt-like immature cells in juvenile rainbow trout responded to amino acids and bile salts. However, mature crypt cells in adult rainbow trout responded strongly to the gonadal extracts from the
opposite sex (Bazáes & Schmachtenberg, 2012). Based on these findings, it appears that arsenic is one of the first chemicals found that impairs crypt cells and their ability to respond to pheromones.

Embryonic-only arsenic exposure delays proliferation and differentiation of neural stem cells in the olfactory epithelium

After observing changes in response to various odorants as functional tests for olfactory sensory neurons, we also investigated the presence of specific OSNs along with numbers of neuronal stem cells and proliferating cells using immunohistochemistry. Crypt cells were unable to be analyzed due to a lack of specific antibodies that reacted with killifish epitopes. The results of our study indicate that embryonic-only arsenic exposures cause a delay in cellular proliferation (PCNA) in the epithelium, which is accompanied by a reduction in Sox2 (neuroblasts) and calretinin (ciliated OSN) expression long after the exposure is removed.

During early development of the olfactory system, olfactory sensory neurons are produced through expression of transcription factors such as foxg1, neurog1, neurod4, and csrc4b, which is followed by cellular migration and proliferation within the olfactory placode region (Miyasaka et al., 2013). Basal stem cells located in the epithelium then differentiate into microvillus, ciliated, or crypt cells for functional tasks of responding to specific odorants. Throughout the lifetime of both fish and mammals, adult neurogenesis is ongoing in the olfactory epithelium for renewal and to regeneration (Cheetham, Park, & Belluscio, 2016; Lindsey et al., 2014). Recovery of damaged OSNs has been seen in zebrafish two weeks after chemical lesioning and exposures to zinc sulfate (Hentig & Byrd-Jacobs, 2016; White et al., 2015). The inability of these stem cells to
appropriately proliferate and differentiate throughout adulthood could lead to reductions in sensory output in response to various odorants.

We assessed the population and function of these stem cells in the olfactory epithelium from early life to adulthood in killifish to determine if arsenic induced an injury early on and if these structures were able to regenerate over the lifetime of the organisms. When correlating PCNA and Sox2 to killifish response to pheromones and TCA, higher levels of PCNA and Sox2 both correlate with greater responses in control fish. Our results show decreased levels of PCNA and Sox2 due to arsenic exposure, as these changes appear immediately after hatching. However, by week 16, there is a dramatic increase in cellular proliferation that continues through week 28. While Sox2 expression, a marker of early neuroblasts, was decreased from hatching through week 16, by week 28, Sox2 expression is also significantly increased. While the PCNA and Sox2 levels seem to be compensating and catching up by week 28, the responses to pheromones and TCA never seem to catch up to the controls. These reductions drive significant differences in IHC-behavior correlations between controls and arsenic groups. These trends indicate proliferation of neural stem cells followed by early differentiation via Sox2 expression, and arsenic seems to delay proliferation and growth of the olfactory epithelium and potentially reduce the ability of killifish to respond to the odorants. This data also suggests that embryonic arsenic exposure impairs differentiation into the neuroblast lineage, but the fish are able to recover as they enter adulthood. Interestingly, expression of calretinin, the marker for ciliated cells, and expression of G\textsubscript{ai3}, the microvillus cell marker, were not altered at hatching. When comparing calretinin levels at each time point to the week 0 control, we see a delay in the increase of calretinin until about week 28. This is because neural stem cells must first proliferate, differentiate into neuroblasts, and then further differentiate into specific olfactory sensory neurons. Our results show that while exposed groups do increase calretinin expression as they
age, calretinin expression is still reduced in the exposed groups compared to the control at later time points. However, correlating TCA response to calretinin expression reveals significant differences between control and exposed groups driven by both reduced responses and reduced calretinin expression, neither of which catches up to the controls by week 28. Comparisons between amino acids responses and PCNA, Sox2, and $G_{\alpha i3}$ did not have as strong trends, which could possibly indicate that arsenic targets ciliated cell types more so than microvillus cells. These results also indicate that embryonic arsenic exposure impairs neuroblast formation and also affects differentiation of a specific olfactory cell type, the ciliated cell.

While almost nothing is known about arsenic’s effect on olfactory development and function, many studies have looked at the effects of arsenic on sensory neurons. For example, correlations were seen between urinary arsenic levels and reduced reaction time in school children, in addition to reduced taste and smell abilities (Calderón et al., 2001; Rodríguez-Barranco et al., 2016; Rosado et al., 2007; Tsuji, Garry, Perez, & Chang, 2015). Adult exposures to arsenic have similar effects on sensory function, with reports of sensory neuropathy in the extremities, visuospatial skills, and reduced touch and vibration sensations (Hafeman et al., 2005; Mukherjee et al., 2003; O’Bryant, Edwards, Menon, Gong, & Barber, 2011). In vivo studies in animals have shown similar effects on neurosensory function, where in utero exposures to arsenic altered auditory responses and reduced sensorimotor reflexes (Gumilar et al., 2015; Xi, Sun, Wang, Jin, & Sun, 2009). When assessing molecular changes in rats, one group found arsenic exposure reduced the number of neural progenitor cells and new mature neurons (S. Liu et al., 2012). Further, arsenic has been shown to impair neuronal differentiation in vitro. Arsenic exposure causes reductions in transcription factors such as neuroD and neurogenin1 in embryonic stem cells, thereby inhibiting differentiation into sensory neurons (Hong & Bain, 2012; J.-T. Liu & Bain, 2018; McCoy, Stadelman, Brumaghim, Liu, & Bain, 2015). Other studies have shown that
arsenic reduces neurite outgrowth and neuronal migration (Maekawa et al., 2013; Zhou et al., 2015). Based on epidemiological, in vivo, and in vitro studies, it is clear that neural progenitor cells are targets of arsenic toxicity that lead to neurobehavioral and neurodevelopmental deficits.

Conclusion

In summary, embryonic-only exposure to arsenic alters proliferation and differentiation of stem cells in the olfactory epithelium as seen by increased PCNA expression and reduced Sox2 and calretinin expression that persists through 28 weeks of age. These changes appear to indicate a delay in cellular division followed by reduced differentiation ability of stem cells. Behavioral changes were seen through 40 weeks of age in response to TCA and pheromones support the lack of functional, olfactory sensory neurons. Overall, these results suggest that stem cells in the olfactory epithelium are a potential target of arsenic toxicity and that adult neurogenesis is likely affected as well.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References


Szymkowicz, D. B., Sims, K. C., Castro, N. M., Bridges, W. C., & Bain, L. J. (2017). Embryonic-only arsenic exposure in killifish (Fundulus heteroclitus) reduces growth and alters muscle IGF levels one year later. *Aquatic Toxicology*.


Figure 4.1. Embryonic arsenic exposure increases start time and reduces killifish response to pheromones. At each time point, 1 mL of adult killifish pheromone extract was added to the top of the water column, and fish response was recorded for 60 seconds. Start time (time for 10% of fish in tank to begin responding) and percentage of fish responding were determined for each exposure (n = 7). Data is presented as start time for weeks 0-40 (A) and percent response for weeks 0-40 (B). Statistical differences were determined using ANOVA and Tukey’s post-hoc test ($p < 0.05$).
Figure 4.2. Embryonic arsenic exposure increases start time and reduces killifish response to TCA through 40 weeks of age. At each time point, 4mL of 250mM TCA was added to the top of the water column, and killifish behavior was recorded for 120 seconds. Start time and percent response were determined for each exposure (n = 7). Data is presented as start time for weeks 0-40 (A) and percent response for weeks 0-40 (B). Statistical differences were determined using ANOVA and Tukey’s post-hoc test (p < 0.05).
Figure 4.3. Embryonic arsenic exposure increases start time and reduces killifish response to amino acids through 40 weeks of age. At each time point, 4mL of a 250mM amino acid mixture was added to the top of the water column, and killifish behavior was recorded for 60 seconds. Start time and percent response were determined for each exposure (n = 7). Data is presented as start time for weeks 0-40 (A) and percent response for weeks 0-40 (B). Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test ($p < 0.05$).
Figure 4.4. Representative confocal images of week 0 immunohistochemistry of olfactory epithelia. Three fish per tank were euthanized and olfactory epithelial regions were collected, formalin-fixed, paraffin-embedded, and sectioned at 5 µm. Tissue sections were stained for PCNA (green; A, D), Sox2 (red; A, D), calretinin (B, E), and \( G_{\alpha i3} \) (C, F). Slides were imaged using a Leica SPE confocal microscope (PCNA, Sox2) and a Leica SP8X confocal microscope (calretinin, \( G_{\alpha i3} \)).
Figure 4.5. Representative confocal images of week 28 immunohistochemistry of olfactory epithelia. Three fish per tank were euthanized and olfactory epithelial regions were collected, formalin-fixed, paraffin-embedded, and sectioned at 5 µm. Tissue sections were stained for PCNA (green; A, D), Sox2 (red; A, D), calretinin (B, E), and Gαi3 (C, F). Slides were imaged using a Leica SPE confocal microscope (PCNA, Sox2) and a Leica SP8X confocal microscope (calretinin, Gαi3).
Figure 4.6. Embryonic arsenic exposure increases PCNA expression and reduces Sox2 in the olfactory epithelium through week 28. Following immunohistochemical staining, the number of cells expressing PCNA (A) or Sox2 (B) were counted and averaged across exposures (n = 4-6) at week 0, 4, 16, and 28. Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test (p < 0.05).
Figure 4.7. Embryonic arsenic exposure decreases calretinin and $G_{\alpha i3}$ expression in the olfactory epithelium through week 28. Following immunohistochemical staining, ImageJ was used to calculate the integrated density of calretinin (A) or $G_{\alpha i3}$ (B) for each sample and averaged across exposures ($n = 4-6$) at week 0, 4, 16, and 28. Calretinin data is presented as fold change of the control at week 0, and $G_{\alpha i3}$ data is present as fold change of the control at each week. Statistical differences (*) were determined using ANOVA and Tukey’s post-hoc test ($p < 0.05$).
Figure 4.8. Correlations between PCNA/Sox2 and percent response to pheromones. Average number of PCNA-expressing cells for each exposure (n = 4-6) at each time point was plotted against average percent (%) response for pheromone behavior tests for each exposure (n = 7) for weeks 0-28 (A-D). Average number of Sox2-expressing cells for each exposure (n = 4-6) at each
time point was plotted against average percent (%) response for pheromone behavior tests for each exposure (n = 7) for weeks 0-28 (E-H). Statistical differences (*) were determined using MANOVA.
Figure 4.9. Correlations between PCNA/Sox2 and percent response to TCA. Average number of PCNA-expressing cells for each exposure (n = 4-6) at each time point was plotted against average percent (%) response for TCA behavior tests for each exposure (n = 7) for weeks 0-28 (A-D). Average number of Sox2-expressing cells for each exposure (n = 4-6) at each time point
was plotted against average percent (%) response for TCA behavior tests for each exposure (n = 7) for weeks 0-28 (E-H). Statistical differences (*) were determined using MANOVA.

Figure 4.10. Correlations between calretinin and percent response to TCA. Average integrated density value for calretinin for each exposure (n = 4-6) at each time point was plotted against average percent (%) response for pheromone behavior tests for each exposure (n = 7) for weeks 0-28 (A-D). Statistical differences (*) were determined using MANOVA.
**Figure S4.1.** Representative confocal images of week 4 immunohistochemistry of olfactory epithelia. Three fish per tank were euthanized and olfactory epithelial regions were collected, formalin-fixed, paraffin-embedded, and sectioned at 5 µm. Tissue sections were stained for PCNA (green; A, D), Sox2 (red; A, D), calretinin (B, E), and $G_{\alpha i3}$ (C, F). Slides were imaged using a Leica SPE confocal microscope (PCNA, Sox2) and a Leica SP8X confocal microscope (calretinin, $G_{\alpha i3}$).
Figure S4.2. Representative confocal images of week 16 immunohistochemistry of olfactory epithelia. Three fish per tank were euthanized and olfactory epithelial regions were collected, formalin-fixed, paraffin-embedded, and sectioned at 5μm. Tissue sections were stained for PCNA (green; A, D), Sox2 (red; A, D), calretinin (B, E), and Gαi3 (C, F). Slides were imaged using a Leica SPE confocal microscope (PCNA, Sox2) and a Leica SP8X confocal microscope (calretinin, Gαi3).
Figure S4.3. Correlations between PCNA/Sox2 and percent response to amino acids.

Average number of PCNA-expressing cells for each exposure (n = 4-6) at each time point was plotted against average percent (%) response for amino acid behavior tests for each exposure (n = 7) for weeks 0-28 (A-D). Average number of Sox2-expressing cells for each exposure (n = 4-6) at
each time point was plotted against average percent (%) response for amino acid behavior tests for each exposure (n = 7) for weeks 0-28 (E-H). Statistical differences (*) were determined using MANOVA.

Figure S4.4. Correlations between Gαi3 and percent response to amino acids. Average integrated density value for Gαi3 for each exposure (n = 4-6) at each time point was plotted against average percent (%) response for amino acid behavior tests for each exposure (n = 7) for weeks 0-28 (A-D). Statistical differences (*) were determined using MANOVA.
CHAPTER FIVE

CONCLUSION

Previous studies in our lab have shown that embryonic-only exposures to 800-5000 ppb arsenic reduced growth in killifish until 16 weeks of age (D’Amico, Gibson, & Bain, 2014). Therefore, we wanted to investigate the effects of more environmentally-relevant arsenic levels on muscle and olfactory development both immediately after the exposure and further into adulthood after the exposure has been long removed. The results from Chapter 2 indicate that arsenic does reduce the overall size of killifish exposed to 200 and 800 ppb arsenic at 16 weeks of age, and these changes persist through at least 40 weeks. While no differences were seen in muscle fiber density, skeletal muscle expression of IGF-1 and its receptor, IGF-1R, were increased through 52 weeks. This suggests that arsenic-exposed organisms increase the local production of IGF-1 as a potential mechanism of compensatory growth. Chapter 3 investigated another possible mechanism by which arsenic alters myogenesis early on in development, specifically through the function of muscle satellite cells. In fish exposed embryonically to arsenic, skeletal muscle satellite cell proliferation was increased at all time points examined. However, when forcing muscle satellite cells to proliferate and differentiate through an induced injury, arsenic exposure significantly impaired the function of these stem cells. Such changes in the function of stem cells not only reduced the ability to regenerate muscle tissue but could also significantly impair initial production of skeletal muscle fibers during embryogenesis and early in life, thereby reducing growth and size. Finally, Chapter 4 investigated the function of neural stem cells in the olfactory epithelium after embryonic-only arsenic exposures to determine if arsenic also affects both developmental and adult neurogenesis. We found that arsenic impairs behaviors associated with ciliated and crypt cells of the olfactory epithelium, and these changes correlated strongly with delayed proliferation and reductions in both neural stem cells and ciliated cells.
One of the major concerns associated with adverse prenatal environments is the long-term effects on the health of individuals and the development of adult diseases. This idea was initially started by Sir David Barker after epidemiology studies associated geographical location with infant mortality and ischemic heart disease (Wadhwa, Buss, Entringer, & Swanson, 2010). The “Barker hypothesis” was further developed to suggest that fetal conditions such as undernutrition and maternal/paternal conditions would predispose individuals for non-communicable adult diseases. This concept was further developed on an international level and coined as the Developmental Outcomes of Health and Disease (DOHaD). It now includes examining multiple aspects of the prenatal and postnatal environments, including chemical exposure, stress, infections, maternal diets, and metabolic status (Rosenfeld, 2017). Due to the high level of developmental plasticity in the fetus, exposure to any one of these conditions can significantly alter birth outcomes such as birth weight and weight gain. For example, high levels of maternal psychosocial stress were associated with insulin resistance and altered immune, endocrine, and cognitive function (Wadhwa et al., 2010). Further, increased cardiovascular disease, chronic kidney disease, and type II diabetes in adults have all been associated with low birthweight (Luyckx & Brenner, 2015; Smith et al., 2016). Fetal exposure to environmental contaminants and endocrine disruptors such as bisphenol-A, phthalates, and heavy metals can also lead to the development of such adult diseases (Heindel, 2018). Because of the relevance of arsenic as an in utero developmental toxicant, it is important to determine whether early exposure can potentially lead to diseases later in life.

Many epidemiological studies have shown that in utero exposures to arsenic have led to reduced birth weight and weight gain, along with neurodevelopmental abnormalities such as reduced IQ, impaired memory function and cognition, and reductions in sensory motor learning (Rahman et al., 2009; Roy et al., 2011; Saha et al., 2012; Tsuji, Garry, Perez, & Chang, 2015).
Most of these epidemiological studies are limited in terms of long-term effects because tracking individuals from cohorts into adulthood becomes difficult. They also focus on continuous arsenic exposure, and little data exists that focuses on embryonic-only exposures. The goal of this dissertation was therefore to expose our model organism, *Fundulus heteroclitus*, to comparable levels of arsenic in contaminated maternal drinking water to mimic only *in utero* exposure, so that we could determine how arsenic impairs both myogenesis and neurogenesis and assess if these changes are permanent after the exposure has been removed.

One of the main consequences of developmental arsenic toxicity is significant reductions in birth weight and head/chest circumference. For example, girls with U-As levels of 26-46 ppb were 300g lighter and 0.7cm shorter at 21 months of age (Saha et al., 2012). Further, children age 4-15 in Bangladesh had significantly lower BMIs in correlation with increasing U-As (Watanabe et al., 2007). Based on the Barker hypothesis, such changes in size and the inability to increase growth in a healthy manner can lead to metabolic deficits and adult diseases. For example, the Women’s Health Initiative cohort reported associations between low birth weight and cardiovascular disease (Smith et al., 2016). In addition, low birth weight has also been associated with central obesity accompanied with the metabolic syndrome (Syndrome X), insulin resistance, and hypertension (Oken & Gillman, 2003). In terms of arsenic exposure, most epidemiological studies only report changes in birth weight or reductions in growth through 4-5 years of age, rather than following a cohort out to adulthood. One of the benefits of our study design was that we were able to assess changes in growth in killifish up to one year in age. This species is sexually mature by approximately 28 weeks of age, so we were able to compare changes in growth into adulthood. At 16 weeks of age, our results showed reductions in growth in fish exposed to 200 and 800 ppb arsenic, and these changes persisted through 40 weeks of age. Long-
term reductions in growth indicate that arsenic is targeting some aspect of growth that is not necessarily recoverable immediately after the exposure has been removed.

The IGF growth pathway has been identified as one of the major pathways for muscle growth in developing individuals, especially in both humans and in fish. Due to its importance in early proliferative growth, the IGF pathway is one potential target of arsenic toxicity that could alter muscle development. One cohort of children in Bangladesh showed negative correlations between urinary arsenic and plasma IGF levels at 4.5 years of age (Ahmed et al., 2013). Similarly, transplacental arsenic exposure in mice resulted in downregulation of the IGF-1 pathway (Xie et al., 2007). And acute arsenic exposure to C2C12 myoblast cells resulted in hypermethylation of the IGF-1 promoter leading to reduction in myogenin, Ptgis and Mef2 (myotube formation genes) expression (Hong & Bain, 2012b).

While we were unable to look at plasma IGF levels in the killifish, we could look at its expression in skeletal muscle. Interestingly, we found a dose-dependent increase in skeletal muscle IGF-1 mRNA expression in killifish at all weeks examined. In concordance with the epidemiological studies, hepatic expression of IGF and IGFBP transcripts from the same fish were suppressed at 8 and 16 weeks in arsenic-exposed fish (Sims et al. 2018; submitted). However, by 28 and 40 weeks, hepatic IGF and IGFBP transcript levels were also overproduced in the arsenic-exposed fish. These results indicate that hepatic IGF production is initially impaired, which would then drive overproduction in skeletal muscle to compensate.

One key difference in typical epidemiological studies and ours is the source of IGF. Most studies measure IGF circulating in blood plasma, whereas we looked at skeletal muscle IGF. Because the IGF pathway is a crucial growth mechanism in both humans and in other species, including fish, upregulation of both plasma IGF and localized IGF (i.e. skeletal muscle) seems to
be common in compensatory growth conditions (Hornick, Van Eenaeme, Gérard, Dufrasne, & Istasse, 2000). Because of the many roles that the IGF pathway plays in growth and proliferation, changes in key components such as IGF-1, IGF-1R, and various IGFBPs can have not only compensatory effects but also detrimental ones. For example, while high level of IGF-1 and IGF-1R are important for normal cellular growth, prolonged overexpression of these proteins can lead to the development of colorectal, breast, and liver cancers (Adamek & Kasprzak, 2018; Giovannucci et al., 2000). High IGF-1 serum levels have also been correlated with pregnancy loss in euploid embryo transfers in rats (Irani, Nasioudis, Witkin, Gunnala, & Spandorfer, 2018). In contrast, brief increases in IGF-1 expression during refeeding and compensatory growth periods have been reported in fish and other animals (Chauvigné, Gabillard, Weil, & Rescan, 2003; Hornick et al., 2000). Elevated IGF-1R mRNA levels have also been associated with obesity in children (Ricco et al., 2018). Increased expression of IGFBPs have been associated with reduced muscle mass in the elderly, in addition to increases in congestive heart failure (Kaplan et al., 2008; Stilling et al., 2017) Because our study appears to be one of the few that shows an upregulation of both IGF-1 and IGF-1R after arsenic exposure as compensation for growth deficits, further analysis of protein expression in skeletal muscle post-exposure may provide important insight for the potential recovery after the exposure is gone.

*In vitro* exposures have shown that arsenic does alter proliferation and differentiation of stem cells via transcriptional alterations in various tissue systems, but there are few *in vivo* studies that address similar effects on myogenesis and neurogenesis. Transcription factors involved in myogenesis such as myogenin, myoD, and mrf4 are reduced after exposure to arsenic, which results in an impairment of myogenic differentiation (Hong & Bain, 2012a; Yen et al., 2010). Previous studies in our lab reported that mouse myoblasts exposed to arsenite had increased methylation of myogenin promoters, leading to decreases in myogenin expression (Hong & Bain,
Further, arsenic exposure to P19 embryonic stem cells lead to suppressed differentiation through the reduction of myogenic transcription factors such as pax3, myoD, myf5, and myogenin (Hong & Bain, 2012a). It appears that arsenic is targeting specific transcription factors involved in myogenesis, therefore preventing proper development of muscle cells. In using an animal model, we wanted to assess muscle satellite cell function after direct injury to skeletal muscle tissue. In control killifish, an induced injury produced a rapid and large increase in proliferating muscle satellite (stem) cells. A week later, during the resolution phase, proliferating cell numbers were reduced to almost control levels. However, there was a dampened response in the fish exposed embryonically to arsenic. Proliferation early after injury was not increased to nearly the same extent as in control fish, and it did not resolve as quickly during the injury recovery phase. Further, we noticed increased levels of collagen in the arsenic exposed fish, indicating a shift towards a fibrotic recovery rather than myogenic regeneration in these fish. Similar findings of enhanced collagen deposition have also been reported in mice models after arsenic exposure (Zhang et al., 2016).

Fibrosis and the production of collagen by myofibroblasts is initially important for remodeling after tissue injury, and under normal circumstances it is eventually replaced with new skeletal muscle tissue. However, if there are alterations in the activity of myofibroblasts, if the ability to produce mature muscle fibers is impaired, or if inflammatory responses persist, collagen may accumulate in the basement membrane of muscle fibers and alter extracellular matrix environments and cellular responses (Grounds, 2014; Karsdal et al., 2017). Long-term deposits of collagen and fibrotic scarring can lead to muscle atrophy, weakness, and overall reductions in skeletal muscle function (Cholok et al., 2017). Because we were limited with numbers of fish to use in the injury study, we were unable to quantify transcript and protein levels of myogenic factors involved in muscle regeneration after an induced injury. However, this would provide
insight as to which part of myogenesis is affected by arsenic and enable one to investigate potential therapies for reduced muscle growth and regeneration, such as myoblast or stem cell therapies used for Duchenne muscular dystrophy (Grounds, 2014).

In addition to reductions in transcription factors needed for myogenesis, another study found that arsenic significantly reduced expression of Pax3, Msx1, Sox10, and neuroD1 in P19 embryonic stem cells, all of which are crucial for the differentiation of neural plate border cells (McCoy, Stadelman, Brumaghim, Liu, & Bain, 2015). Neural plate border specifier cells are produced early on in development as progenitors of the neural crest. These cells receive various signals (Wnt, Notch, Bmp) to further differentiate into either myogenic or neural progenitor cells, one of the initial steps in the formation of skeletal muscle cells and sensory neurons, respectively (Betancur, Bronner-Fraser, & Sauka-Spengler, 2010; Noisa et al., 2014). In addition to its negative effects on myogenesis both in vitro and in vivo, arsenic is also known to impact neurogenesis. Previous studies in our lab determined that arsenic exposure reduced P19 stem cell differentiation into neural stem cells by repressing the expression of key neuronal transcription factors such as neurogenin1, neurogenin2, and neuroD (Hong & Bain, 2012a). While there is plenty of in vitro evidence supporting arsenic’s effects on neural stem cell function, in vivo studies are much more limited. Further, there are no current studies to date that assess the effects of arsenic on sensory neurons, such as those in the olfactory system, both immediately after exposure and into adulthood. The olfactory epithelium is a unique structure that undergoes constant turnover of olfactory sensory neurons, making it an ideal model for adult neurogenesis. In killifish, we were able to determine that embryonic-only arsenic inhibits proper neurogenesis later in life by permanently impairing the function of neural stem cells. Through various odorant response tests, we found that behaviors in response to bile salts and pheromones were altered well into adulthood. These changes were accompanied by reductions in neuroblast populations and
ciliated olfactory sensory neurons, in addition to delays in cellular proliferation. Because similar processes occur in neurogenesis in other regions of the brain, arsenic’s effects on stem cells could have detrimental consequences in other pathways of neurodevelopment. While assessing protein markers for these cell types through immunohistochemistry is only semi-quantitative, it may be extremely useful to look at actual protein levels and function of the receptors on the olfactory sensory neurons to further explore the target of arsenic toxicity.

In conclusion, our studies were developed in order to determine if embryonic-only arsenic exposure has permanent effects on skeletal muscle and sensory neuron development. After using concentrations that are commonly found in maternal drinking water, including the EPA drinking water standard of 10 parts per billion, our results suggest that this current drinking water standard is not completely protective of developmental health, especially in regard to neurodevelopment. Another aspect that needs to be considered is the possibility for arsenic exposure from multiple sources, especially food sources such as rice (Davis et al., 2012; Lynch, Greenberg, Pollock, & Lewis, 2014). We reported effects on muscle development at 50 ppb arsenic, and changes in behavior and neurodevelopment were also seen at 10 ppb, which is the current standard. Because of the neurodevelopmental risks of arsenic exposure and the levels found worldwide in rice, rice milk, and rice cereal, there appears to be a need for additional restrictions on arsenic content in food sources. Inorganic arsenic levels of 130μg/kg have been reported in rice and 92 μg/kg in infant rice cereals (Lynch et al., 2014). Recommended limits of arsenic in rice have been set at 200μg/kg for white rice and 100μg/kg for infant and children’s food in the EU, but these limits are only under consideration in the U.S. (Cubadda, Jackson, Cottingham, Van Horne, & Kurzius-Spencer, 2017). Further, probabilistic modeling reported the mean exposure to inorganic arsenic in food is approximately two times higher than that of background drinking water levels in the U.S., with rice being one of the largest contributors. Therefore, further exploration of these
mechanisms and the extent of arsenic toxicity during *in utero* exposures is important for determining what the drinking water standard and potentially food standards should be set at to protect developmental health.
References


